University of Alberta

Isolation and Characterization of Mutations Affecting Alternative Oxidase Regulation in *Neurospora crassa*

by

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Abstract

When the cytochrome mediated electron transport chain in the fungus *Neurospora crassa* is disrupted, an alternative oxidase, encoded by the nuclear gene *aod-1*, is induced. The alternative oxidase donates electrons directly to oxygen from ubiquinone, bypassing two proton pumping sites of the normal electron transport chain.

Little is known about the control of *aod-1* expression. To facilitate isolation of mutations affecting the regulation of the gene, a tyrosinase based reporter system was constructed. The reporter construct contains the region upstream of the *aod-1* gene fused to the coding sequence of the *N. crassa* enzyme tyrosinase, so that expression of the enzyme is controlled by signals that normally affect *aod-1*. Expression of tyrosinase is easily monitored, as its activity turns colonies brown when they are overlaid with a tyrosine solution. The reporter construct was integrated into the genome of a strain shown to carry a null allele of the endogenous gene encoding tyrosinase.

When cells containing the reporter system were mutagenized and assayed for tyrosinase activity under conditions that normally induce alternative oxidase, eighteen mutant strains were isolated that affected induction of both the reporter and endogenous alternative oxidase. Complementation analysis revealed that four novel loci involved in *aod-1* regulation, named *aod-4*, *aod-5*, *aod-6*, and *aod-7*, had been isolated. Mutations in these genes were shown to prevent induction of *aod-1* mRNA and protein. An *aod-4* strain also exhibited a temperature-sensitive growth defect at 30°C and was deficient in cytochromes aa_3 and *b*. The *aod-4* gene maps to the right arm of linkage group V.

A chloramphenicol resistant mutation (*chl-1*) was also isolated. Chloramphenicol prevents mitochondrial translation, thereby disrupting oxidative phosphorylation and

inducing alternative oxidase. Unlike wild-type strains grown in the presence of chloramphenicol, cells carrying the *chl-1* mutation have normal levels of cytochromes aa_3 and *b*, and do not induce alternative oxidase activity. However, growth of the *chl-1* strain in the presence of the complex III inhibitor antimycin A does induce alternative oxidase activity. The *chl-1* gene maps to the right arm of linkage group II, and is the only chloramphenicol resistance marker reported for *N. crassa* to date.

Dedicated to my family, my friends, and Dr. Chris Todd.

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Abbreviations

A. chrysogenum	*Acremonium chrysogenum*
A. italicum	*Arum italicum*
A. maculatum	*Arum maculatum*
A. nidulans	*Aspergillus nidulans*
A. thaliana	*Arabidopsis thaliana*
3-AT	3-amino-1,2,4-triazole
AAC	adenosine 5'-diphosphate adenosine 5'-triphosphate carrier
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
amp	ampicillin
arc	aerobic respiratory control
ATP	adenosine 5'-triphosphate
β-Gal	β-galactosidase
BAC	bacterial artificial chromosome
bHLH/Zip	basic helix-loop-helix zipper
BLAST	basic local alignment search tool
C. albicans	*Candida albicans*
C. elegans	*Caenorhabditis elegans*
C. parapsilosis	*Candida parapsilosis*
°C	degrees Celsius
cAMP	cyclic adenosine 5'-monophosphate
m-CLAM	*m*-chlorobenzhydroxamic acid
CORR	co-localization for redox regulation
CRE	cyclic adenosine 5'-monophosphate response element
CREB	cyclic adenosine 5'-monophosphate response element binding protein
D. melanogaster	*Drosophila melanogaster*
2-D	two-dimensional
ddNTP	dideoxynucleotide 5'-triphosphate
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5'-triphosphate
Dopa	dihydroxyphenylalanine
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
EMS	ethyl methanesulfonate
EPR	electron paramagnetic resonance
ER	endoplasmic reticulum
$FAD/FADH_2$	flavine adenine dinucleotide
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
FMN	flavine mononucleotide
G. graminis	*Gaeumannomyces graminis*

G. max	*Glycine max*
g	grams
GFP	green fluorescent protein
GIP	general insertion pore
GMP	guanine 5'-monophosphate
gRNA	guide ribonucleic acid
GUS	β-glucouronidase
H. capsulatum	*Histoplasma capsulatum*
HOG	high osmolarity glycerol
hr	hours
Hsp	heat shock protein
IMS	intermembrane space
IPG	immobilized pH gradient
kan	kanamycin
kb	kilobases
kbp	kilobase pairs
KCN	potassium cyanide
kDa	kiloDalton
L	liter
LB	Luria-Bertani
LG	linkage group
M. atropurpureum	*Macroptilium atropurpureum*
M. grisea	*Magnaporthe grisea*
M. indica	*Mangifera indica*
μg	micrograms
μL	microliters
M	molar
MALDI-TOF	matrix-assisted laser desorption ionization – time of flight
mA	milliamp
MAPK	mitogen activated protein kinase
Mbp	megabase pairs
MBSU	Molecular Biology Service Unit
MDa	megaDalton
MIM	mitochondrial inner membrane
min	minutes
mL	milliliter
mM	millimolar
MNNG	*N*- methyl-*N*'-nitro-*N*-nitrosoguanidine
MnSOD	manganese superoxide dismutase
MOM	mitochondrial outer membrane
MPP	matrix processing peptidase
mRNA	messenger ribonucleic acid
msec	milliseconds
mtDNA	mitochondrial deoxyribonucleic acid
N. crassa	*Neurospora crassa*
N. tabacum	*Nicotiana tabacum*

NAD/NADH + H^+	nicotinamide adenine dinucleotide
NEM	*N*-ethylmaleimide
nm	nanometers
ori	origin of replication sequences
OD	optical density
ORF	open reading frame
P. anomala	*Pichia anomala*
P. anserina	*Podospora anserina*
P. stipitis	*Pichia stipitis*
PCR	polymerase chain reaction
P_i	inorganic phosphate
pI	isoelectric point
PR	pathogenesis related
PSI	pounds per square inch
PT	permeability transition
ρ^-	large deletion of mitochondrial deoxyribonucleic acid
ρ^0	no mitochondrial deoxyribonucleic acid
RIP	repeat induced point mutation
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*
S. foetidus	*Symplocarpus foetidus*
S. guttatum	*Sauromatum guttatum*
S. lipolytica	*Saccharomyces lipolytica*
S. pombe	*Schizosaccharomyces pombe*
spp.	species
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHAM	salicylhydroxamic acid
SOD	superoxide dismutase
SSF-126	(*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl)acetamide
T. brucei	*Trypanosoma brucei*
TCA	tricarboxylic acid cycle
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
UAS	upstream activation site
URF	unidentified open reading frame
UV	ultraviolet
V	volt
Vhr	volt hour
V. radiata	*Vigna radiata*

1 Introduction

1.1 Mitochondria

Mitochondria are double membrane-bound organelles found in almost all eukaryotic organisms. Of the few eukaryotes that lack mitochondria, some contain hydrogenosomes, which are thought to have evolved from the same ancestor (Bui *et al.*, 1996; Dyall and Johnson, 2000), while others contain genetic vestiges that suggest they once had mitochondria that have since been lost (Lang *et al.*, 1999). Well known as the site of energy production by oxidative phosphorylation, mitochondria also play a role in iron and calcium homeostasis, β-oxidation, amino acid biosynthesis, apoptosis and cellular aging. Mitochondria are not created *de novo* but are formed by growth and division of pre-existing organelles. Mitochondrial morphology and distribution can vary in different cell types or at different developmental stages (Yaffe, 1999; Jensen *et al.*, 2000; Shaw and Nunnari, 2002; Westermann, 2002). From the traditional bean shape, to elongated tubes, to a branching reticulum, mitochondria can assume more than one external conformation. They can be evenly distributed throughout the cell or localize to specific subcellular regions.

1.2 Mitochondrial origin

Mitochondria are thought to have arisen from an endosymbiotic event, a model of which is known as the serial endosymbiosis hypothesis (Gray *et al.*, 1999; Andersson *et al.*, 2003; Doolittle *et al.*, 2003; Allen, 2003). The proto-mitochondrion is thought to have been a free-living α-proteobacterium that was engulfed by an anaerobic archaebacterium, which may or may not have already had a true nucleus. Mitochondria still contain remnants of this lineage, as their genetic system resembles that of bacteria and several mitochondrial proteins appear to have a proteobacterial origin. Initially it was thought that the endosymbiont offered the host energy in exchange for a favorable environment. However it seems unlikely that a free-living proteobacterium would have an adenosine triphosphate (ATP) excretion system for releasing energy. A syntrophic hypothesis has been suggested with the endosymbiont providing hydrogen to the host in exchange for host-produced methane (Martin and Muller, 1998; Moreira and Lopez-Garcia, 1998). The

ATP generating pathway of the endosymbiont may also have detoxified the increasingly abundant oxygen of the host's environment (Andersson and Kurland, 1999). Oxygen maintained a selective pressure on the host to keep the proto-mitochondrion, and on the proto-mitochondrion to keep the energy pathway. Over time, proto-mitochondria lost much of their ancestral genome. There would be no selective pressure to maintain redundant genes, and the essential proto-mitochondrial genes were transferred to the nucleus. Gene transfer may have been favoured because of the selective pressure described as Muller's ratchet: in asexually inherited genomes, deleterious mutations accumulate because they cannot be removed or repaired through recombination (Race *et al.*, 1999; Blanchard and Lynch, 2000).

1.3 Mitochondrial DNA (mtDNA)

Despite the large gene transfer that occurred over evolution, mitochondria maintain their own genome, and there are several theories for why certain genes have not been transferred. One hypothesis suggests that gene transfer is ongoing and eventually all mitochondrial proteins will be encoded in the nucleus (Adams *et al.*, 2000; Doolittle *et al.*, 2003). However, the subset of genes that are currently present in mtDNA across a range of species is not random, suggesting that selective pressure has maintained certain genes in the mitochondrial genome while allowing others to transfer (Race *et al.*, 1999; Allen, 2003). It has been suggested that mtDNA still encodes proteins that cannot be imported from the cytosol, possibly due to large hydrophobic domains that would hinder passage through mitochondrial membranes, or to a toxic effect that might occur if the proteins were released in the cytosol (von Heijne, 1986; Palmer, 1997; Martin and Herrmann, 1998). In support of this notion, the degree of hydrophobicity of a nuclear encoded mitochondrial protein has been shown to be responsible for allowing or preventing its import (Daley *et al.*, 2002). However, it is also conceivable that cytosolic chaperones could be adopted to allow hydrophobic domains or toxic proteins to correctly import (Race *et al.*, 1999; Allen, 2003). Another theory is known as the hypothesis of co-localization for redox regulation (CORR), which suggests that organellar genomes retained a specific subset of genes whose expression is directly controlled by the redox state of either their gene product or other components with which their gene product interacts (Race *et al.*, 1999; Allen, 2003). Mitochondria would thereby be able to immediately and directly regulate mtDNA gene expression to respond and adapt to changing redox states. The unrelated subset of genes encoding mitochondrial ribosomal RNAs (rRNAs) and other factors for gene expression would have to be retained in the mitochondrial genome to allow synthesis of the CORR gene products within mitochondria. While the transcription of the chloroplast genome encoded photosystem I and II genes is known to be regulated by the redox status of plastoquinone, there is no evidence as yet for CORR in mitochondria. Nevertheless, mtDNA generally encodes oxidative phosphorylation subunits that could be subjected to CORR, as well as the rRNAs and in many cases the transfer RNAs (tRNAs) required to translate mtDNA encoded messenger RNAs (mRNAs).

The mitochondrial genome can range in size from less than 6 kbp in apicomplexan *Plasmodium* species to over 2400 kbp in certain higher plants (Backert *et al.*, 1997; Gray *et al.*, 1999; Nosek and Tomaska, 2003). Many species also contain mitochondrial plasmids, and both plasmids and the main mitochondrial genome can be maintained as either circular or linear molecules depending on the species (Nosek *et al.*, 1998; Nosek and Tomaska, 2003).

1.3.1 *Neurospora crassa* mitochondrial DNA

Fungal mtDNA ranges in size from 18 to 167 kbp (Backert *et al.*, 1997) and can be compact or contain large non-coding regions (Paquin *et al.*, 1997). The linear or circular mtDNA encodes 7 to 14 oxidative phosphorylation subunits, 2 rRNAs, a variable number of tRNAs, and may contain unidentified open reading frames (URFs). mtDNAs from some fungi, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, do not encode subunits for complex I of oxidative phosphorylation. Both group I and group II introns and mobile elements have been seen in fungal mtDNA.

The mtDNA in *N. crassa* is a circular molecule 64.84 kbp in length (Griffiths *et al.*, 1995; Kennell *et al.*, in press). It encodes 2 rRNAs, 27 tRNAs, 5 URFs, and 15 polypeptides, including 7 complex I subunits, apocytochrome *b* of complex III, 3 complex IV subunits, 3 complex V subunits, and a ribosomal subunit called S5. In various natural isolates, different combinations of group I and group II introns can be found, as well as linear and circular plasmids. Integration of certain plasmids into the mtDNA can cause senescence, a cessation of growth thought to be caused by disruption of oxidative phosphorylation (Griffiths *et al.*, 1995; Kennell *et al.*, in press). Many mtDNA mutations have been described and certain deletions, insertions and point mutations can be suppressive, such that extended growth or sub-culturing leads to the predominance of mutant mtDNA over wild-type. Suppressive mutations affect oxidative phosphorylation and cause a three-fold increase in number of mitochondria seen by electron microscopy (EM; Bertrand, 1995). The preponderance of mutant mtDNAs could be due to a replicative advantage of a mtDNA with a large deletion, or a bias in gene conversion favouring the mutant sequence (Bertrand, 1995; Griffiths *et al.*, 1995; Kennell *et al.*, in press). Functionally impaired mitochondria may also be induced to proliferate in response to their reduction in oxidative phosphorylation resulting in an increase in mutant mtDNA over wild-type (Bertrand, 1995).

1.3.2 Mitochondrial DNA structure and inheritance

Studies on mtDNA replication, maintenance and inheritance have focused on *S. cerevisiae* since this yeast is a facultative aerobe and can survive when oxidative phosphorylation is disrupted, enabling isolation of mtDNA mutants (Contamine and Picard, 2000). Yeast *petite* mutants have been described that contain either large deletions of mtDNA, called ρ^-; or no mtDNA, called ρ^0. In *S. cerevisiae*, mtDNA has been shown to associate with proteins in a complex called the nucleoid (Kaufman *et al.*, 2000; Birky, 2001). Approximately 100 mtDNA molecules per cell are organized into 10 to 20 nucleoids, in which the mtDNA may be held together by Holliday junctions created during replication and recombination. Nucleoids have been suggested to be attached to the mitochondrial inner membrane (MIM), an arrangement that may be required for proper mtDNA transmission to daughter cells (Nunnari *et al.*, 1997; Contamine and Picard, 2000; Hobbs *et al.*, 2001). When mitochondria from both parents fuse in the zygote, their proteins intermix but their mtDNA remain localized to distinct regions, resulting in reduced heteroplasmy in daughter cells (Nunnari *et al.*, 1997).

1.4 Mitochondrial protein import

The majority of mitochondrial proteins are encoded in the nucleus, synthesized on cytosolic ribosomes as precursor proteins, and imported into mitochondria (reviewed in Bauer *et al.*, 2000; Kunau *et al.*, 2001; Truscott *et al.*, 2003; Rehling *et al.*, 2003; Hoppins *et al.*, in press). These precursor proteins have to be sorted to the correct sub-compartment: the mitochondrial outer membrane (MOM), the MIM, the intermembrane space (IMS) or the matrix. Studies of the import and sorting of precursors have been done primarily using *S. cerevisiae* and *N. crassa* as models. Most precursor proteins have an N-terminal targeting presequence 10 to 80 residues long that contains basic, hydrophobic and hydroxylated amino acids that can form an amphipathic α-helix with positive amino acids on one side and hydrophobic residues on the other (Roise *et al.*, 1988; Lemire *et al.*, 1989; Abe *et al.*, 2000). The presequence is cleaved by the matrix processing peptidase (MPP) during or after import (Luciano and Geli, 1996). Further sorting signals can follow the presequence and may also be cleaved by MPP or by the inner membrane peptidase (Nunnari *et al.*, 1993). Other precursor proteins do not have a recognizable presequence; rather, they carry their import and sorting signals internally within the mature protein sequence (Brix *et al.*, 1999; Wiedemann *et al.*, 2001). Various cytosolic chaperones may bind the precursor to maintain an import competent conformation (Murakami *et al.*, 1988; Hachiya *et al.*, 1993).

Only one import complex has been described for the MOM, the translocase of the outer membrane (TOM) complex. The TOM complex facilitates the translocation of precursor proteins across or into the MOM, and consists of a general insertion pore (GIP), or core complex composed of the more tightly associated components Tom40[1], Tom22, Tom7, Tom6 and Tom5, as well as the more loosely associated Tom70 and Tom20 subunits (Dekker *et al.*, 1998; Ahting *et al.*, 1999). Tom70, Tom22 and Tom20 have large cytosolic domains, and Tom70 and Tom 20 act as receptors for initial precursor recognition. Tom70 is a receptor for precursors that contain no presequence (Brix *et al.*, 1999; Wiedemann *et al.*, 2001) and may also be a docking site for cytosolic chaperones (Komiya *et al.*, 1997; Young *et al.*, 2003). Tom20 is the receptor for presequence containing precursors (Brix *et al.*, 1999; Abe *et al.*, 2000). Tom70 and Tom20 then pass precursor proteins to the secondary receptor, Tom22, which facilitates the insertion of precursors into the GIP (Kiebler *et al.*, 1993). Receptor dependent binding of precursor proteins on the cytosolic side of the MOM is said to occur at a position called the *cis* binding site. As precursors traverse the TOM complex pore, they bind the *trans* site on the IMS side of the TOM complex (Mayer *et al.*, 1995). Tom40 is the major component of the TOM complex and is thought to make up the pore through which precursors pass (Hill *et al.*, 1998). Tom5 aids in correctly positioning the precursor for translocation and may act as the receptor for small Tim proteins (Dietmeier *et al.*, 1997). Tom6 helps stabilize Tom40 and the receptors (Alconada *et al.*, 1995), while Tom7 destabilizes the Tom40/receptor interaction (Hönlinger *et al.*, 1996).

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[1] Numerals following the "Tom" designation indicate the molecular weight of an individual TOM complex component (Pfanner *et al.*, 1996).

Once the precursor has bound the *trans* site, it can go on to interact with one of two translocases of the inner membrane (TIM) complexes. The interaction of a precursor at the *trans* binding site with either TIM complex causes contact sites between the MOM and the MIM (Schleyer and Neupert, 1985). Pores through the MIM must be tightly regulated to prevent leakage of ions that would dissipate the chemiosmotic gradient essential for energy formation, and interaction of the presequence in the presence of membrane potential stimulates pore activation (Bauer *et al.*, 1996; Truscott *et al.*, 2001; Kovermann *et al.*, 2002). The TIM23 complex is generally responsible for sorting precursors containing presequences into the matrix, while the TIM22 complex deals with precursors with internal sorting signals that are destined for insertion into the MIM. The TIM23 complex consists of Tim23[1], Tim17, Tim44, and Tim50. Subunits Tim23 and Tim17 are homologous, and either Tim23 alone (Truscott *et al.*, 2001) or both Tim23 and Tim17 (Milisav *et al.*, 2001) are thought to form the pore of the TIM23 complex. In *S. cerevisiae*, the N-terminus of Tim23 may insert into the MOM, suggesting a role for this protein in keeping the TIM23 complex in close proximity to the MOM to facilitate rapid precursor transfer (Donzeau *et al.*, 2000). Tim44 is peripherally associated with the TIM23 complex on the matrix side of the MIM (Moro *et al.*, 1999). Tim44 associates with the chaperone heat shock protein (Hsp) mtHsp70 that binds to the precursor as it traverses the pore. mtHsp70 may either generate an ATP derived force required to pull the precursor, as described in the motor or pulling model, or may act as an anchor to prevent retrograde movement of the precursor, as in the Brownian ratchet or trapping model (Neupert and Brunner, 2002).

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[1] Numerals following the "Tim" designation indicate the molecular weight of an individual TIM complex component (Pfanner *et al.*, 1996).

The TIM22 complex consists of Tim22, Tim18, Tim54 and Tim12. Soluble complexes consisting of Tim9 and Tim10, or Tim8 and Tim13 direct transfer of the precursor from the TOM complex to the TIM22 complex (Koehler *et al.*, 1998a; Koehler *et al.*, 1998b; Paschen *et al.*, 2000; Curran *et al.*, 2002). These complexes associate with the precursor and may stimulate its release from the TOM complex, shield hydrophobic domains from the aqueous environment of the IMS, and/or guide the precursor to the TIM22 complex. The carrier class of proteins, including the adenosine diphosphate ADP/ATP carrier (AAC), utilizes the Tim9-Tim10 complex in the IMS (Koehler *et al.*, 1998a; Koehler *et al.*, 1998b; Curran *et al.*, 2002). Under conditions of low membrane potential, insertion of Tim23 itself requires the Tim8-Tim13 complex (Paschen *et al.*, 2000). Tim22 makes up the two pores of the TIM22 complex (Kovermann *et al.*, 2002) and has homology to Tim23 and Tim17 (Sirrenberg *et al.*, 1996).

At least one more translocase exists in the MIM to insert precursors from the mitochondrial matrix into the MIM. This process is called mitochondrial export because it occurs in the opposite direction as mitochondrial protein import (Truscott *et al.*, 2003; Hoppins *et al.*, in press). These precursors are either nuclear-encoded proteins that have gone through both the TOM and TIM23 complexes to the matrix, or mitochondrially encoded proteins synthesized in the matrix. Mitochondrial protein export can be achieved via the Oxa1 translocase protein, which has homology to bacterial inner membrane proteins and a chloroplast thylakoid membrane protein involved in insertion of proteins into membranes (Stuart, 2002). Oxa1 is required for insertion of several membrane proteins, but it remains unclear whether Oxa1 proteins form a pore (Hell *et al.*, 2001; Truscott *et al.*, 2003). There may be other matrix exporting complexes beyond the OXA translocase that have yet to be identified.

1.5 Mitochondrial function

Several enzymes of the urea cycle and arginine biosynthetic pathways are localized to the mitochondrial matrix (reviewed in Wu and Morris, 1998; Morris, 2002). Arginine is a precursor for the synthesis of proline and glutamate, a compound involved in the tricarboxylic acid (TCA) cycle. Mitochondria are also the major site for β-oxidation, the breakdown of fatty acids to produce energy (reviewed in Eaton *et al.*, 1996; Eaton, 2002). Enzymes in the MIM and the matrix synthesize acetyl-CoA during fatty acid breakdown, a process that generates reducing equivalents directly passed to ubiquinone or stored in reduced nicotinamide adenine dinucleotide (NADH + H^+). Acetyl-CoA is used as a substrate of the TCA cycle, producing more cellular energy.

Mitochondria are also involved in controlling the homeostasis of iron and calcium ions in the cell. Iron ions are incorporated in the mitochondria into heme groups and iron-sulfur clusters used to catalyze electron transfer (reviewed in Lill and Kispal, 2000; Mühlenhoff and Lill, 2000; Atamna *et al.*, 2002; Foury and Kucej, 2002). Calcium ions are important in cellular signaling and in regulating processes such as transcription, cell proliferation, muscle contraction, neurotransmitter release, pancrease secretion, fertilization and apoptotic cell death (reviewed in Pozzan *et al.*, 2000; Berridge *et al.*, 2000; Breckenridge *et al.*, 2003; Carafoli, 2003; Roderick *et al.*, 2003). Calcium ion release from the endoplasmic reticulum (ER) can create localized regions of high calcium concentration called microdomains. The close proximity of mitochondria and ER observed microscopically (Manella *et al.*, 1998) may allow mitochondria to act as a buffering system to control the amplitude and spread of the calcium signal.

Apoptosis is vital for multicellular development and homeostasis because it allows removal of cells without the toxic release of cellular contents. Mitochondria have been described as scaffolds for assembly of apoptosis controlling factors in *Caenorhabditis elegans*, and are known to be direct regulators of apoptosis in higher animals (reviewed in Desagher and Martinou, 2000; Green, 2000; Waterhouse, 2003; Newmeyer and Ferguson-Miller, 2003). Bcl-2 family members in higher animals are key regulators of apoptosis, hypothesized to control mitochondrial fragmentation during apoptosis. These proteins may facilitate mitochondrial protein release by destabilizing the MOM, opening the permeability transition (PT) pore, or oligomerizing to form a pore.

Mitochondria release cytochrome *c*, which then binds apoptotic protease activating factor and causes its oligomerization into the caspase activating apoptosome (Liu *et al.*, 1996; Zou *et al.*, 1997; Cain *et al.*, 2000). Smac/DIABLO protein is also released from mitochondria and joins the apoptosome to inhibit a caspase inhibitor (Du *et al.*, 2000; Verhagen *et al.*, 2000). Another protein released from mitochondria, apoptosis inducing factor, translocates to the nucleus and initiates chromatin condensation and DNA fragmentation (Susin *et al.*, 1999).

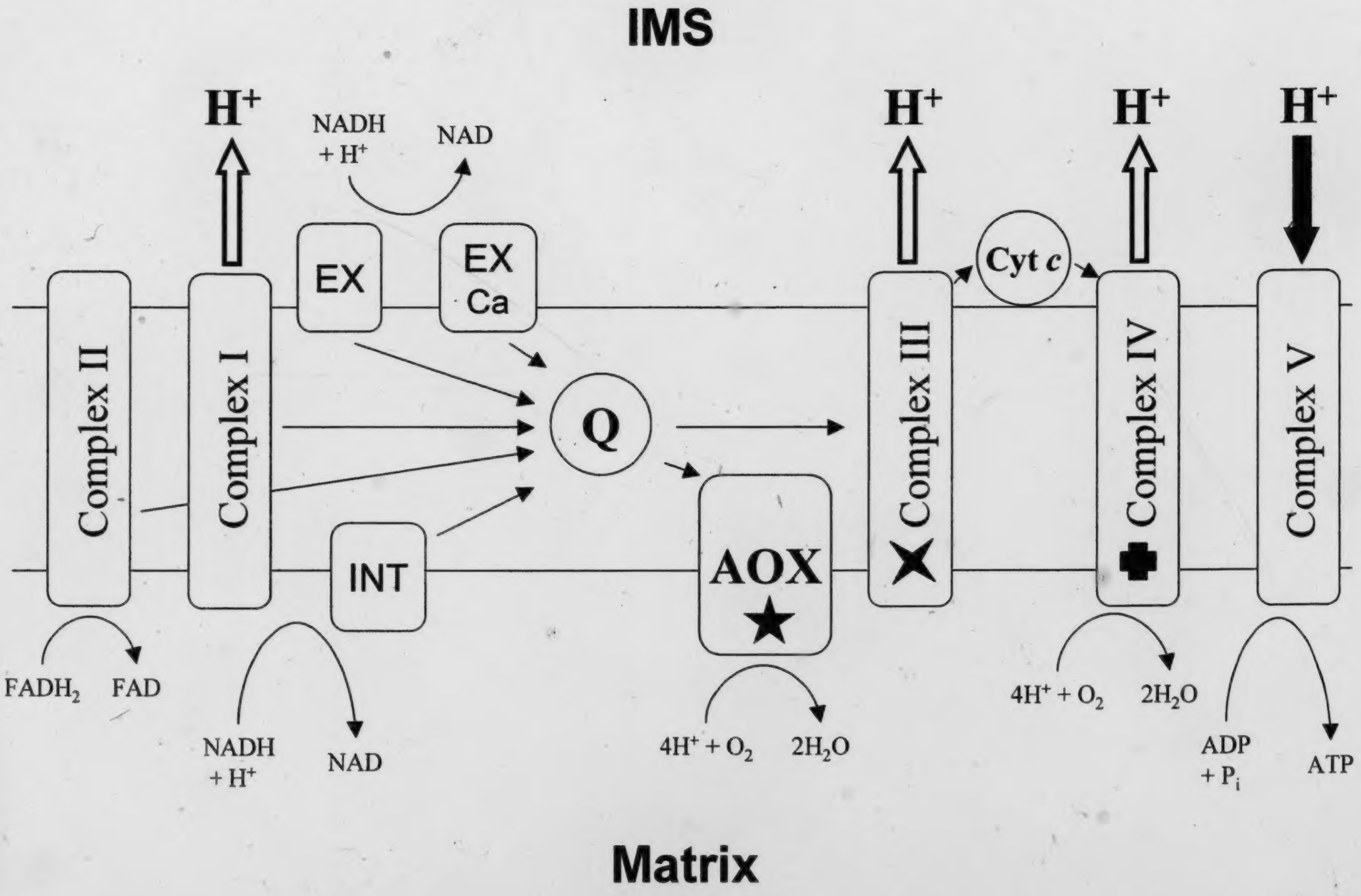
Mitochondrial have also been implicated in aging (reviewed in Mandavilli *et al.*, 2002; Brunk and Terman, 2002; de Grey, 2002a; de Grey, 2002b; Wei and Lee, 2002; Sastre *et al.*, 2003; Hekimi and Guarente, 2003). The mitochondrial theory of aging suggests that mitochondria accumulate damage to mtDNA, proteins and lipids from exposure to reactive oxygen species (ROS) created during oxidative phosphorylation. This damage and the resulting reduction in energy production can target cells for apoptosis. Multiple cell death events over time cause a decrease in organ function, and eventually organism death. The *clk-1* gene product in *C. elegans* is involved in ubiquinone biosynthesis, and *clk-1* mutants do not contain the normal isoform of ubiquinone, but use a ubiquinone precursor as an electron carrier (Ewbank *et al.*, 1997; Miyadera *et al.*, 2001). Mutations of *clk-1* slow development and increase life span while maintaining the same ATP level as wild-type. Mutation of *mev-1*, a subunit of complex II in *C. elegans*, causes increased ROS production and premature aging (Senoo-Matsuda *et al.*, 2001). Again, ATP levels are the same in this mutant as in wild-type. Finally, mutations in *isp-1*, an iron-sulfur cluster subunit of complex III in *C. elegans*, reduce ROS production and slow development and aging (Hekimi and Guarente, 2003). These mutants demonstrate a correlation between aging and ROS production, but not necessarily between aging and a decrease in energy levels.

A role for ROS scavenging systems in controlling aging has been investigated in *C. elegans*, *Drosophila melanogaster*, and *Mus musculus*. Mutations in the *C. elegans* genes *age-1* (Larsen, 1993; Yanase *et al.*, 2002) and *daf-1* (Honda and Honda, 1999) that increase life span also increase the activity of manganese superoxide dismutase (MnSOD), a mitochondrial enzyme involved in removing ROS. In *D. melanogaster*, mutations in catalase, an enzyme that converts hydrogen peroxide to water, cause a decrease in life span (Griswold *et al.*, 1993). Deletions of the SOD genes in mice have also been shown to decrease life span (Li *et al.*, 1995; Melov *et al.*, 1999). Homozygous knock out mice rarely survive past 7 days after birth, and heterozygotes have increased mtDNA damage and exhibit a swift aging-like decline. Thus, the role of mitochondria in aging may be limited to generation and removal of cytotoxic ROS, rather than induction of apoptosis due to declining energy production or accumulation of mtDNA mutations.

Deficiencies in mitochondrial function are found in many human diseases (reviewed in Wallace, 1999; Simon and Johns, 1999; Graff *et al.*, 2002; Di Mauro *et al.*, 2002; Khrapko *et al.*, 2003; Rossignol *et al.*, 2003). Mitochondrial disease is estimated to occur in 1 in 10, 000 live births, though the frequency of involvement of mtDNA mutations in many common diseases may be more widespread than predicted (Chinnery and Turnbull, 2001). Mitochondrial diseases exhibit a wide phenotypic variability due to varying mutational loads and threshold effects, and it is hard to relate specific mtDNA mutations with clinical symptoms. The phenotype of a given mutation can also vary with age, and many diseases show delayed onset. This delay could be due to the additive effect of general mitochondrial decline seen with aging, or changing mutational loads that occur during mitotic segregation of mtDNA. Environmental factors, and nuclear and mitochondrial genetic factors may also play a role in the disease penetrance.

1.6 Oxidative phosphorylation

Mitochondria are the site of oxidative phosphorylation, the process by which energy from electron transfer generates a chemiosmotic gradient used to produce ATP (reviewed in Hatefi, 1985; Saraste, 1999; Joseph-Horne *et al.*, 2001; Schultz and Chan, 2001). During glycolysis and the TCA cycle, electrons are transferred to the carriers NADH + H^+ and flavine adenine dinucleotide ($FADH_2$), which then pass the electrons on to the respiratory chain (Fig. 1). Four complexes in the MIM make up the electron transport chain: complex I, the NADH:ubiquinone oxidoreductase; complex II, the succinate:ubiquinone oxidoreductase; complex III, the ubiquinone:cytochrome *c* oxidoreductase; and complex IV, the cytochrome *c*:oxygen oxidoreductase. Complex V constitutes the ATP synthase and is made up of the F_0 portion in the MIM, and the peripheral membrane proteins of the F_1 portion on the matrix side of the MIM. Electrons


IMS
H+
H+
H+
H+
NADH + H+
NAD
EX
EX Ca
Cyt c
Complex II
Complex I
Q
Complex III
Complex IV
Complex V
INT
AOX
FADH2
FAD
NADH + H+
NAD
4H+ + O2
2H2O
4H+ + O2
2H2O
ADP + Pi
ATP
Matrix

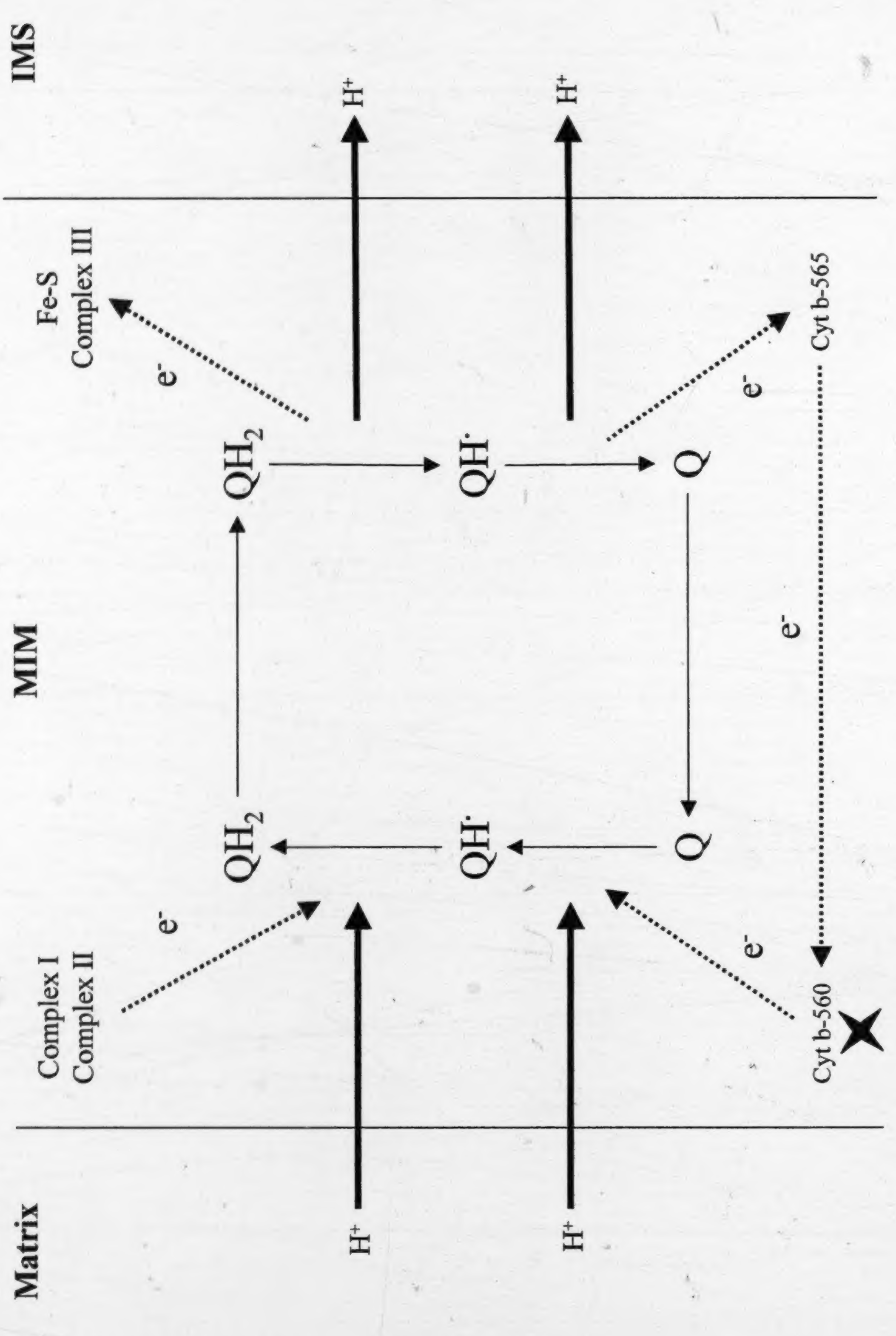
move from complex I or complex II to ubiquinone, then to complex III, to cytochrome *c*, and finally to complex IV, where electrons are donated to molecular oxygen with four hydrogen ions to produce two molecules of water. As electrons flow through the redox centers of complexes I, III and IV, their potential energy is used by the complexes to drive the unidirectional movement of hydrogen ions from the matrix to the IMS. This pumping of hydrogen ions creates a chemiosmotic gradient across the MIM, the potential energy of which is harvested by complex V.

Complex I has been well studied in *N. crassa* (Videira, 1998; Videira and Duarte, 2002). The complex is made up of about 35 peptide subunits, 32 of which have been identified, and has a molecular weight of approximately 1 MDa. Electrons are passed by eight iron-sulfur clusters, a flavin mononucleotide (FMN), and an as yet unidentified redox center thought to be unique to *N. crassa* (Joseph-Horne *et al.*, 2001; Videira and Duarte, 2002). The flow of one electron through complex I is estimated to pump two hydrogen ions (Joseph-Horne *et al.*, 2001; Schultz and Chan, 2001). EM studies of complex I from *N. crassa* reveal that the complex is L-shaped, with a membrane arm in the MIM and a peripheral arm in the matrix (Guénebaut *et al.*, 1998). The redox centers are all found on the peripheral arm, which also contains the binding site for NADH + H^+. The membrane arm is thought to form a channel for hydrogen ion pumping and contains at least two ubiquinone binding sites (Schultz and Chan, 2001; Videira and Duarte, 2002). Rotenone binds complex I at these ubiquinone sites, thus preventing electron flow (Earley *et al.*, 1987). The seven subunits encoded by *N. crassa* mtDNA are positioned in the membrane arm (Videira, 1998; Videira and Duarte, 2002). In the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis (Schlünzen *et al.*, 2001), a smaller complex I assembles that is thought to consist of only the peripheral arm (Friedrich *et al.*, 1989; Schultz and Chan, 2001).

Bacteria, plants, and fungi (including *N. crassa*) also contain alternative dehydrogenases that transfer electrons from NADH + H^+ to ubiquinone without pumping hydrogen ions (Kerscher, 2000; Joseph-Horne *et al.*, 2001). In fact, *S. cerevisiae*, *Saccharomyces carlsbergii*, *S. pombe*, and *Kluyveromyces lactis* have no complex I, and rely entirely on alternative NADH dehydrogenases. These dehydrogenases are rotenone insensitive and can accept electrons from NADH + H^+ in either the matrix (internal) or the IMS (external). *N. crassa* has at least one internal and two external NADH dehydrogenases. One of the external NADH dehydrogenases can bind, and may be regulated by, calcium ions (Videira and Duarte, 2002). When the electron transport chain is highly reduced, the alternative dehydrogenases are thought to prevent ROS formation at complex I (Kerscher, 2000; Joseph-Horne *et al.*, 2001).

Complex II is part of the TCA cycle and converts succinate to fumarate, coupled to the reduction of FAD to $FADH_2$. Electrons from $FADH_2$ are then passed to ubiquinone with no associated hydrogen ion pumping (Hägerhäll, 1997). Complex III contains cytochromes c_1 and *b*, exists as a dimer, and uses the ubiquinone cycle to pass electrons and shuttle hydrogen ions to the IMS (Fig. 2; Mitchell, 1979; Brandt and Trumpower, 1994; Zhang *et al.*, 1998). Oxidized ubiquinone located on the matrix side of complex III is reduced by two electron uptake events, each of which is coupled to the binding of a hydrogen ion from the matrix. One electron is donated by cytochrome b_{560} while the other comes from complex I or II. Reduced ubiquinol then moves to the IMS side of complex III where it is oxidized in two stages, each of which allows the release of a hydrogen ion to the IMS. The first oxidation event donates an electron to an iron-sulfur cluster, causing a conformational change in the iron-sulfur containing protein (Zhang *et al.*, 1998). The hydrophilic IMS domain of this subunit rotates, taking the cluster away from ubisemiquinone. The iron-sulfur cluster donates this electron to cytochrome c_1, which passes the electrons to the IMS soluble cytochrome *c*. The movement of this complex III subunit forces ubisemiquinone to donate the second electron to cytochrome b_{565}. Fully oxidized ubiquinone then moves back to the matrix side of the complex, and the second electron is passed from cytochrome b_{565} to cytochrome b_{560} to begin the cycle again (Mitchell, 1979; Brandt and Trumpower, 1994). Antimycin A binds to cytochrome b_{560}, thereby blocking electron flow through the ubiquinone cycle (Zhang *et al.*, 1998).

Reduced cytochrome *c* is oxidized by complex IV, which pumps one hydrogen ion for every electron (Michel *et al.*, 1998). Complex IV has four redox centers consisting of two copper ions and the cytochromes *a* and a_3. One of the copper ions and cytochrome a_3 form a binuclear site responsible for transferring electrons to oxygen to produce water. The inhibitor cyanide binds cytochrome a_3 and prevents electron flow (van Buuren *et al.*, 1972). Complex IV is thought to have at least two channels for



Matrix
MIM
IMS
H+
H+
H+
H+
Complex I
Complex II
Fe-S
Complex III
e-
e-
e-
e-
e-
QH2
QH·
Q
QH2
QH·
Q
Cyt b-560
Cyt b-565

hydrogen ion pumping (Michel *et al.*, 1998), and conformational changes caused by electron flow through the complex result in the unidirectional movement of hydrogen ions through these channels (Schultz and Chan, 2001).

Complex V harnesses the energy of the chemiosmotic gradient by coupling hydrogen ion flow to the production of ATP (Boyer, 1997). Subcomplex F_0 is embedded in the MIM and forms the channel for hydrogen ion flow back to the matrix. Connected to F_0 by a stalk, the F_1 subcomplex is the site of ATP formation. The flow of hydrogen ions makes the γ subunit of F_1 rotate, causing conformational changes that regulate binding of ADP and inorganic phosphate (P_i), and release of ATP (Noji *et al.*, 1997).

1.7 Alternative oxidase

In addition to the cytochrome pathway, higher plants, non-fermenting fungi, algal species and trypanosomes also contain an alternative pathway of respiration (Fig. 1; Henry and Nyns, 1975; Lambers, 1982; McIntosh, 1994; Vanlerberghe and McIntosh, 1997; Siedow and Umbach, 2000; Joseph-Horne *et al.*, 2001). Electron flow through the alternative pathway is insensitive to antimycin A, cyanide, carbon monoxide and nitric oxide, but is inhibited by iodoacetate, fluoride (James and Beevers, 1950), thiocyanate, 8-hydroxyquinoline, α,α'-dipyridyl (Bonner *et al.*, 1967), aromatic hydroxamic acids such as salicylhydroxamic acid (SHAM) or *m*-chlorobenzhydroxamic acid (*m*-CLAM; Schonbaum *et al.*, 1971), disulfiram (Grover and Laties, 1978), and gallate esters (Stegink and Siedow, 1986).

Use of specific inhibitors of the cytochrome and alternative pathway was crucial for the elucidation of the order and kinetics of the different electron carriers (Storey and Bahr, 1969a). *Symplocarpus foetidus* mitochondria were isolated from the reproductive spadix tissue, which was known to exhibit high levels of alternative respiration. ADP/oxygen ratios for these mitochondria in the presence or absence of cyanide revealed that alternative respiration bypassed two sites of phosphorylation and did not involve the cytochromes (Storey and Bahr, 1969b). The oxidation rates of ubiquinone and flavoproteins were unaffected by the addition of cyanide to mitochondria from *S. foetidus*, or to mitochondria isolated from etiolated hypocotelydons of *Phaseolus aureus*, which also have alternative respiration (Storey and Bahr, 1969a; Storey, 1970a). The redox state of ubiquinone in *N. crassa* mitochondria isolated from cultures with alternative respiration was also unaltered by cyanide, and extraction of ubiquinone inhibited both pathways, which suggested that ubiquinone may act as a branch point between the two pathways (von Jagow *et al.*, 1973; von Jagow and Bohrer, 1975). Indeed, ubiquinone oxidation in isolated *S. foetidus* spadix mitochondria in the presence of carbon monoxide was blocked by *m*-CLAM, further proving that ubiquinone is the branch point (Storey, 1976). Electron flow from ubiquinone to the alternative pathway bypasses complexes III and IV, explaining the loss of two sites of phosphorylation (Fig. 1). Thus, alternative respiration does not contribute to the chemiosmotic gradient and can be considered energetically wasteful (Moore *et al.*, 1978). The alternative pathway uses oxygen as the final electron acceptor, forming water (Sharpless and Butow, 1970; Downie and Garland, 1973; Huq and Palmer, 1978; Rich, 1978).

The molecular nature of alternative respiration was unclear for several years because it was spectroscopically indistinct (Bonner *et al.*, 1986). Early studies with electron paramagnetic resonance (EPR) failed to reveal a specific signal for alternative respiration despite the sensitivity of this pathway to metal chelators (Schonbaum *et al.*, 1971; Downie and Garland, 1973; Rich and Bonner, 1978). It was hypothesized that a novel cytochrome or flavoprotein may be involved in this pathway (James and Beevers, 1950; Bendall and Hill, 1956; Storey, 1970a; Storey, 1970b; Erecinska and Storey, 1970; Storey, 1970c). However, redox rates of the cytochromes and flavoproteins of *S. foetidus* spadix mitochondria in the presence of antimycin A were exceedingly slow, ruling out their involvement in alternative respiration (Storey and Bahr, 1969a; Bonner *et al.*, 1967; Bendall and Bonner, 1971). Partial purification of alternative respiration from *Arum maculatum* spadix mitochondria contained a quinol oxidase but no cytochromes (Huq and Palmer, 1978; Rich 1978).

Polyclonal and monoclonal antibodies were raised against a further purified fraction from *Sauromatum guttatum* spadix mitochondria, and identified four polypeptides of 37, 36, 35.5 and 35 kDa associated with alternative respiration (Elthon and McIntosh, 1987; Elthon *et al.*, 1989). These antibodies cross reacted to all four polypeptides, suggesting that they were post-translationally modified or degradation products of the same polypeptide. The antibody recognized related proteins in a variety of plant and fungal species: *Arum italicum*, *Vigna radiata*, *Amorphophallus rivieri*, *S. foetidus* (Elthon and McIntosh, 1987), *Persea americana*, *Solanum tuberosum*, *Nicotiana tabacum* (Elthon *et al.*, 1989), *Glycine max*, *Macroptilium atropurpureum* (Kearns *et al.*, 1992), *Petunia hybrida* (Wagner, 1995), *Poa spp.* (Millenaar *et al.*, 2001), *Mangifera indica* (Considine *et al.*, 2001), *N. crassa* (Lambowitz *et al.*, 1989), *Pichia anomala* (Sakajo *et al.*, 1993), *Gaeumannomyces graminis* (Joseph-Horne *et al.*, 1998), *Candida albicans* (Huh and Kang, 1999), *Pichia stipitis* (Umbach and Siedow, 2000), and *Podospora anserina* (Borghouts *et al.*, 2001). The cross reactivity of the antibodies suggests that alternative oxidase has been conserved throughout diverse species.

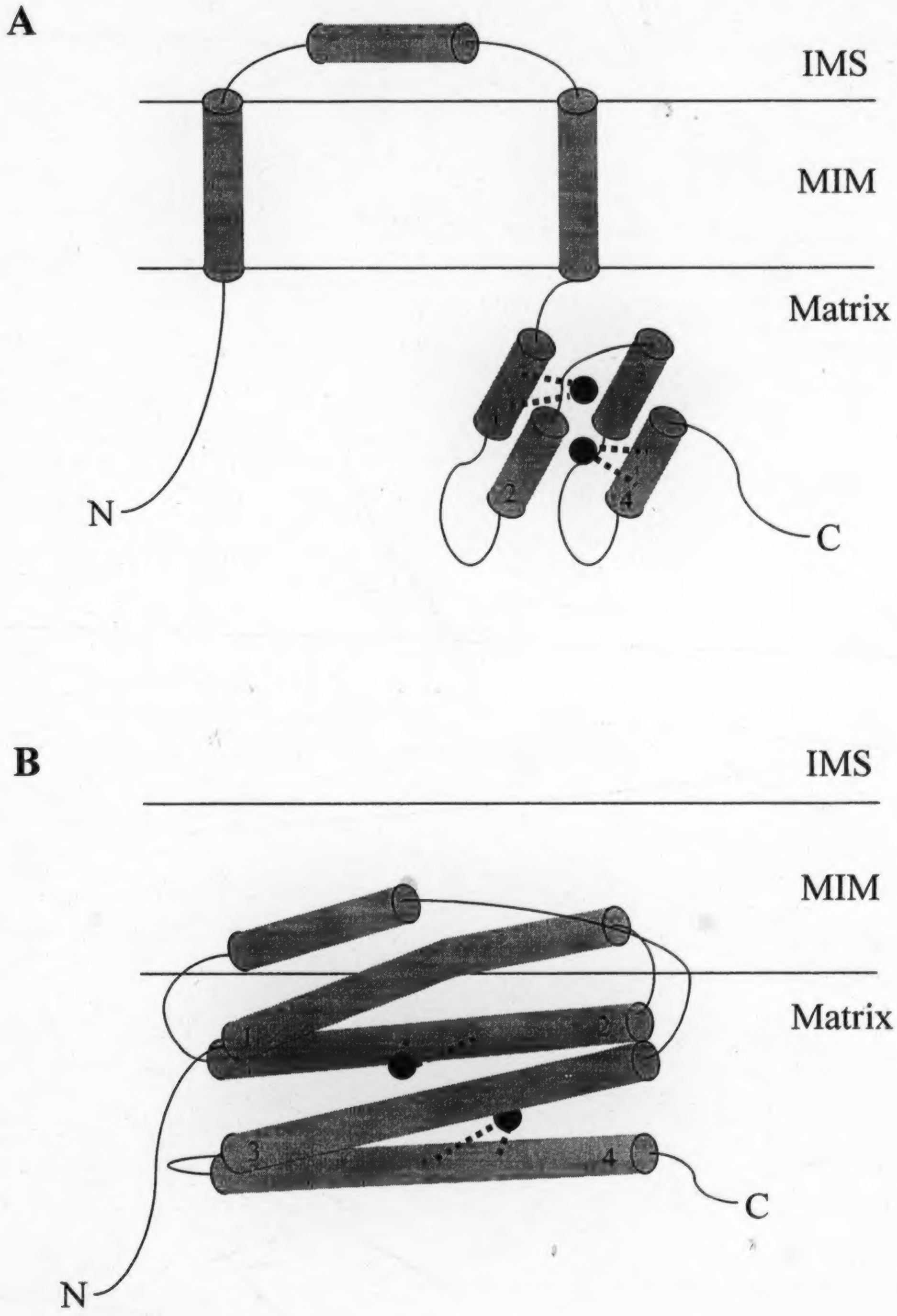
Using the antibody to screen expression libraries, the alternative oxidase gene was cloned from *S. guttatum* (Rhoads and McIntosh, 1991) and *G. max* (Whelan *et al.*, 1993). A *hemA* respiratory mutant of *Escherichia coli* was rescued by the alternative oxidase gene from *Arabidopsis thaliana* (Kumar and Söll, 1992), revealing that alternative respiration can be achieved by a single polypeptide, the alternative oxidase. The gene from *Trypanosoma brucei* was also able to rescue the *E. coli hemA* mutant (Chaudhuri and Hill, 1996), the *S. guttatum* gene conferred cyanide-insensitive respiration to *S. pombe* (Albury *et al.*, 1996), and the *C. albicans* (Huh and Kang, 1999; Huh and Kang, 2001), *P. stipitis* (Shi *et al.*, 2002), and *Histoplasma capsulatum* (Johnson *et al.*, 2003) genes conferred cyanide-insensitive respiration to *S. cerevisiae*.

1.7.1 Alternative oxidase structure

The high levels of cyanide-insensitive respiration present in *A. maculatum* spadices were localized to the mitochondria (James and Elliott, 1955). Trypsin treatment of *A. maculatum* mitoplasts and inside-out submitochondrial particles revealed that alternative oxidase was associated with the MIM and was at least partially exposed to the matrix (Rasmusson *et al.*, 1990). Since alternative respiration can be inhibited by various metal chelators, it was suggested that its activity may involve a novel non-heme iron oxidase (Bonner *et al.*, 1967; Bendall and Bonner, 1971). The requirement of iron for activity was confirmed in *Saccharomyces lipolytica* and *P. anomala*. The appearance and activity of alternative respiration was increased by addition of ferric ions to *S. lipolytica*, while addition of ferrous, cobalt, copper, aluminum, lanthanum, magnesium, and manganese ions had no effect (Henry *et al.*, 1977). Ferrous rather than ferric ions were shown to be necessary for alternative oxidase activity in *P. anomala* (Minigawa *et al.*, 1990).

Short stretches of conserved amino acids from the derived alternative oxidase protein sequences of *S. guttatum*, *A. thaliana*, *G. max*, *N. tabacum*, and *P. anomala* suggested that alternative oxidase may be a binuclear iron protein, due to several conserved E-X-X-H putative iron binding motifs (Fig. 3A; Siedow *et al.*, 1995; Moore *et al.*, 1995). Other binuclear iron proteins containing coupled high-spin iron atoms that do not have an EPR signal when reduced or oxidized had been described, and it was found that partially purified alternative oxidase also had no associated EPR signal. Thus, it was attractive to place alternative oxidase in the same family. A model was proposed in which the N-terminal hydrophobic regions of the enzyme were proposed to span the MIM, and the conserved E-X-X-H motifs were placed on α-helices one and four of a four helix bundle (Siedow *et al.*, 1995; Moore *et al.*, 1995). Weaknesses of this model included relatively short helices, fairly tight turns of only three residues between helices, and the placement of iron interacting residues on helices one and four rather than the usual two and four.

More recently, a revised model was proposed, taking into account the larger body of information that had become available on di-iron carboxylate protein structures (Fig. 3B; Andersson and Nordlund, 1999). A third highly conserved E-X-X-H motif was identified that had been placed in the IMS by the first model. This motif and the more C-terminal E-X-X-H were placed in the second and fourth α-helices to hold the binuclear iron center, an arrangement more in line with current models for other members of the di-iron binuclear family. This model also had more typical length helices and suggested that instead of traversing the MIM, alternative oxidase either bound to an integral MIM protein or was associated with only one lipid layer of the MIM as a monotopic or interfacial integral protein. Discovery of the chloroplast binuclear iron protein IMMUTANS in *A. thaliana* lead to a revision of the second model, rotating the third helix and bringing a conserved tyrosine residue in proximity to the iron center (Berthold *et al.*, 2000). Site directed mutagenesis gave support to the revised second model. Mutation of the glutamate and histidine residues of the significant E-X-X-H motifs of the


A
IMS
MIM
Matrix
1
2
3
4
N
C
B
IMS
MIM
Matrix
1
2
3
4
C
N

revised second model in *T. brucei* alternative oxidase failed to rescue respiration in *hemA E. coli* (Ajayi *et al.*, 2002). Mutation of these glutamates and conserved tyrosine residues in the *A. thaliana* gene in an *S. pombe* background also disrupted cyanide-insensitive respiration (Albury *et al.*, 2002). Moreover, recently developed techniques for acquiring mixed valent EPR signals from coupled binuclear iron proteins revealed that alternative oxidase generates signals characteristic of these proteins (Berthold *et al.*, 2002).

The revised second model also predicted that alternative oxidase formed a hydrophobic pocket from the MIM leading to the iron center that could be a ubiquinone interacting channel (Andersson and Nordlund, 1999). Support for this prediction was obtained when the *A. thaliana* alternative oxidase was subjected to error prone polymerase chain reaction (PCR), and SHAM resistant derivatives that could still rescue the *hemA E. coli* strain were selected (Berthold, 1998). The resistant substitutions were in the predicted hydrophobic pocket, where SHAM could possibly inhibit activity by blocking ubiquinone binding.

1.7.2 Alternative oxidase capacity versus engagement

The cytochrome and alternative oxidase pathways have specific capacities in a given situation, where the term "capacity" is defined as the physical capability for electron flow through each pathway. However, respiratory pathways are not always fully engaged, where "engagement" refers to the degree to which a specific respiratory pathway contributes to overall respiration. Thus, the engagement of one pathway can increase upon addition of inhibitors of the other pathway.

It was believed that the two pathways directly competed for electrons, with some equilibrium at the branch point that favoured flow to the cytochromes (Bahr and Bonner, 1973). Although the branch point was identified as ubiquinone (von Jagow *et al.*, 1973; von Jagow and Bohrer, 1975; Storey, 1976), it was unclear how ubiquinone partitioned electrons between pathways. In mitochondria from *G. max*, electrons did not flow through alternative oxidase until the level of ubiquinone pool reduction was 35-40%, above which alternative respiration was significantly engaged in a non-linear fashion (Dry *et al.*, 1989). This engagement suggested that instead of competing for electrons, the alternative pathway acted as an overflow for electrons when the cytochrome pathway was highly engaged (Lambers, 1980; Lambers 1982; Dry *et al.*, 1989). However, addition of pyruvate to isolated mitochondria from *G. max* reduced the threshold level of ubiquinone reduction required to engage alternative oxidase (Umbach *et al.*, 1994; see section 1.7.3). The presence of pyruvate was shown to divert electrons from the cytochrome pathway to the alternative pathway, which suggested that instead of the overflow model, the two pathways were indeed competing for electrons (Hoefnagel *et al.*, 1995).

Electron partitioning between the two pathways was studied using oxygen isotopes in isolated *G. max* mitochondria (Ribas-Carbo *et al.*, 1997). Alternative oxidase and complex IV have different degrees of discrimination against using the ^{18}O and ^{16}O isotopes of oxygen, so the overall engagement of each pathway can be calculated from the relative amounts of the two isotopes consumed. It was shown that the level of engagement of the alternative pathway depended on the amount of alternative oxidase protein present, the redox state of the dimer of alternative oxidase (see section 1.7.3), the addition of pyruvate (see section 1.7.3), and the size and redox state of the ubiquinone pool. Thus, the engagement of alternative oxidase can be modified by a number of factors, which allows partitioning of electrons between the pathways to be actively regulated and adapted in response to changing metabolic and redox states (Vanlerberghe and McIntosh, 1997).

A two step reduction model was suggested for electron transfer to explain the kinetics of alternative oxidase engagement (Siedow and Moore, 1993). Alternative oxidase was proposed to accept two electrons from ubiquinol, accept two more electrons from another ubiquinol much less readily, and then react with oxygen to form water. An activation step prior to the reduction of oxygen was later included (Ribas-Carbo *et al.*, 1994). However, a difficulty with this catalytic model was that a binuclear iron center would not be expected to be able to carry four electrons (Siedow *et al.*, 1995; Affourtit *et al.*, 2002). Other cycles have recently been suggested. One model proposed that alternative oxidase may accept two electrons from ubiquinol, bind and partially reduce oxygen to a peroxide intermediate, and then receive two more electrons from a second ubiquinol to reduce the peroxide to water (Berthold *et al.*, 2000; Affourtit *et al.*, 2002). Another model predicted that after accepting two electrons at the binuclear center to produce ferric ions, alternative oxidase could bind but not yet reduce oxygen (Affourtit *et* *al.*, 2002). Two more electrons are instead suggested to be accepted at the binuclear center to make ferryl ions, not considered likely for this class of enzyme, or by amino acid(s) proximal to the catalytic site to make redox-active radical residue(s). Then oxygen could be fully reduced in a single step, as is seen in other oxygen metabolizing enzymes (Affourtit *et al.*, 2002).

1.7.3 Alternative oxidase dimerization and regulation by pyruvate

Alternative oxidase can exist as a monomer, a non-covalently associated dimer, or a covalently bound dimer (Umbach and Siedow, 1993). When the reducing agent was removed from the electrophoresis buffer for samples of *G. max*, *S. guttatum*, and *V. radiata* mitochondria, the expected 30 to 39 kDa monomer bands for the alternative oxidase protein were seen, as well as one or more bands between 60 and 75 kDa for each organism (Umbach and Siedow, 1993). The molecular weight of these new bands suggested that they were homodimers possibly covalently bound via a disulfide bond. Addition of a chemical cross linker revealed that the lower band "monomers" were actually associated non-covalently as dimers *in vivo*.

Reduction of the covalent disulfide bond, either by addition of dithiothreitol (DTT) or naturally over the course of development in *S. guttatum*, resulted in increased alternative oxidase activity. The dimer status of alternative oxidase in *G. max* was also directly related to activity levels, where it was revealed that the non-covalently linked dimer was more active than the covalently bound dimer (Umbach *et al.*, 1994). These data supported an earlier suggestion that disulfide bonds or sulfhydryl groups regulated alternative respiration in *Ipomoea batatas*, where inhibition of this pathway by the sulfhydryl reacting compound disulfiram was prevented by the presence of a thiol (Grover and Laties, 1978). The involvement of a disulfide bond was substantiated by treating *G. max* mitochondria with iodoacetate or *N*-ethylmaleimide (NEM), two sulfhydryl reacting compounds (Umbach and Siedow, 1996). These treatments prevented the formation of the covalently linked dimer, while the non-covalently associated dimer was maintained. Iodoacetate stimulated alternative oxidase activity, and the structure of the adduct that iodoacetate was known to form with sulfhydryls suggested that a thiohemiacetal group may be formed.

Two N-terminal cysteine residues in alternative oxidase that could be involved in the disulfide bond between dimer subunits are highly conserved in plant alternative oxidases (Vanlerberghe and McIntosh, 1997). Mutagenesis of these residues to alanines in transgenic plants of *N. tabacum* identified the more N-terminal cysteine as the residue involved in disulfide bond formation (Vanlerberghe *et al.*, 1998). Mutation of the cysteines to alanines in an *A. thaliana* alternative oxidase gene expressed in *E. coli* also pointed to the N-terminal cysteine (Rhoads *et al.*, 1998). It was suggested that the reduction of this disulfide bond may serve as a post-translational mechanism of regulating alternative oxidase activity, with the enzyme most active as a reduced, non-covalently associated dimer (Umbach and Siedow, 1993; Umbach *et al.*, 1994). However, recent studies using *Poa annua* whole root extracts (Millenaar *et al.*, 1998), *A. thaliana* whole leaf extracts (Simons *et al.*, 1999), or mitochondria of *N. tabacum* isolated in the presence of pyruvate (Vanlerberghe *et al.*, 1999) revealed that alternative oxidase is predominantly present as the non-covalently associated dimer regardless of activity level. Thus, the covalent bond seen between alternative oxidase dimers can have a regulatory effect on alternative oxidase activity, but is not the only controlling factor.

The activity of alternative oxidase in plants has been shown to be stimulated by pyruvate and other α-keto carboxylic acids (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Day and Wiskich, 1995; Millar *et al.*, 1996; Umbach and Siedow, 1996; Vanlerberghe *et al.*, 1998; Rhoads *et al.*, 1998; Millenaar *et al.*, 1998; Vanlerberghe *et al.*, 1999; Pastore *et al.*, 2001; Umbach *et al.*, 2002). Studies using mitochondria from *G. max* suggested that pyruvate increased the ability of alternative oxidase to bind ubiquinone (Millar *et al.*, 1993) and reduced the threshold of ubiquinone reduction required to engage alternative oxidase (Umbach *et al.*, 1994). This stimulation of alternative oxidase allowed it to compete directly with the cytochrome pathway for electrons (Hoefnagel *et al.*, 1995; see section 1.7.2). A role for pyruvate in controlling alternative oxidase activity made inherent sense. That is, when respiration through the cytochrome pathway is blocked, the TCA cycle can no longer continue, pyruvate accumulates, and the alternative pathway is activated (Millar *et al.*, 1993; Umbach *et al.*, 1994; Hoefnagel *et al.*, 1995; Day and Wiskich, 1995). In mitochondria from *A. italicum* and *G. max*, pyruvate was shown not to increase the affinity of alternative oxidase for ubiquinol but to increase the amount of active enzyme (Hoefnagel *et al.*, 1997). Treatment of *G. max* mitochondria with iodoacetate and NEM blocked pyruvate stimulation of alternative oxidase activity, which suggested that pyruvate acted at the same site as these reagents and could also form a thiohemiacetal with a cysteine residue (Umbach and Siedow, 1996). Mutation of the same cysteine involved in covalent dimer formation to alanine in *N. tabacum* (Vanlerberghe *et al.*, 1998) or *A. thaliana* (Rhoads *et al.*, 1998) made alternative oxidase activity insensitive to pyruvate activation. Replacing this cysteine with glutamate, in an attempt to mimic the thiohemiacetal adduct, resulted in levels of alternative oxidase activity nearing those of wild-type enzyme in the presence of pyruvate (Rhoads *et al.*, 1998). Thus, the N-terminal conserved cysteine residue can form a disulfide bond that inactivates the dimer, or can form a thiohemiacetal with pyruvate to activate the non-covalently associated dimer (Rhoads *et al.*, 1998). Since alternative oxidase can exist predominantly as a non-covalently associated dimer (Millenaar *et al.*, 1998; Simons *et al.*, 1999; Vanlerberghe *et al.*, 1999), the interaction of pyruvate with this cysteine rather than formation of a disulfide bond may be responsible for regulating alternative oxidase activity (Vanlerberghe *et al.*, 1999).

The mechanism of alternative oxidase activation by pyruvate was suggested to be through preventing oxidized ubiquinone from remaining in the active site of the enzyme (Hoefnagel and Wiskich, 1998). Mutagenesis of the conserved cysteine in the *A. thaliana* gene expressed in *E. coli* revealed that a variety of charged residues were sufficient to maintain alternative oxidase activity, suggesting that a charge repulsion was required (Umbach *et al.*, 2002). Thus, thiohemiacetals produced by pyruvate and cysteine may cause a conformational change through charge repulsion between the subunits of the dimer that is required for activity.

1.7.4 Alternative oxidase activation by mononucleotides

Fungal alternative oxidases do not contain the N-terminal cysteine conserved in plant species (Joseph-Horne *et al.*, 2000). Although the *G. graminis* alternative oxidase has been shown to form a covalently associated dimer (Joseph-Horne *et al.*, 1998), the enzyme from *Magnaporthe grisea* (Yukioka *et al.*, 1998b), *N. crassa* and *P. stipitis* (Umbach and Siedow, 2000) does not. Addition of diamide, which promotes disulfide bond formation, or chemical cross linkers to *N. crassa* and *P. stipitis* mitochondrial samples failed to generate a dimeric sized band, and addition of pyruvate or other α-keto acids had no effect on enzyme activity. Thus, fungal alternative oxidase generally does not exist as a non-covalent or covalent dimer and its activity is not regulated by pyruvate.

Adenosine monophosphate (AMP) stimulated alternative respiration in the fungus *Moniliella tomentosa* (Vanderleyden *et al.*, 1980b) as was previously shown for the protist *Euglena gracilis* (Sharpless and Butow, 1970). Alternative respiration in mitochondria and submitochondrial particles of *N. crassa* grown in the presence of chloramphenicol is stimulated by a variety of mononucleotides (Vanderleyden *et al.*, 1980a; Michea-Hamzehpour and Turian, 1987; Umbach and Siedow, 2000). Purine mononucleotides gave a stronger stimulation than pyrimidine mononucleotides, with guanine monophosphate (GMP) causing the highest activity level. Alternative oxidase activity in mitochondria from *P. anomala* grown in the presence of antimycin A shows activation by purine mono- or diphosphate nucleotides, but not by pyrimidines, cyclic AMP (cAMP), or triphosphate nucleotides (Sakajo *et al.*, 1997). Alternative oxidase activity is also stimulated by AMP and GMP in *Acanthamoeba castellanii* (Jarmuszkiewicz *et al.*, 2001) and by GMP in *Candida parapsilosis* (Milani *et al.*, 2001). Isolated mitochondria from *P. stipitis* showed activation of alternative oxidase by GMP, and it was suggested that the areas of the polypeptide responsible for nucleotide activation in fungi could lie in the N-terminal or extended C-terminal regions that are not conserved between fungi and plants (Umbach and Siedow, 2000).

1.7.5 Regulation of alternative oxidase expression

Alternative oxidase is expressed in a tissue and developmentally specific manner in several plants, including a system of differential expression of members of an alternative oxidase multigene family (Elthon *et al.*, 1989; Kearns *et al.*, 1992; Whelan *et al.*, 1996; Finnegan *et al.*, 1997; Saisho *et al.*, 1997; McCabe *et al.*, 1998; Murcha *et al.*, 1999; Saisho *et al.*, 2001; Considine *et al.*, 2001; Saika *et al.*, 2002; Djajanegara *et al.*, 2002; Karpova *et al.*, 2002). In plants with multigene families, the alternative oxidase genes are believed to serve different roles, with some being involved in housekeeping functions while others are induced by stress (Considine *et al.*, 2002).

Western analysis of isolated mitochondria from *M. atropurpureum* shoots, roots, and from nodules associated with both tissues showed two, one, and no alternative oxidase isoforms, respectively (Kearns *et al.*, 1992). In a similar experiment using isolated mitochondria from *G. max*, cotyledon and leaf mitochondria were shown to have two alternative oxidase bands, root mitochondria, one band, and uninfected nodules, no bands. Isolated mRNA from *G. max* tissues was subjected to *in vitro* translation and immunoprecipitation with alternative oxidase antibody. While a protein product was precipitated from samples where cotyledon mRNA had been used for the translation, no protein was precipitated from samples derived from the uninfected nodule tissue. This indicated that tissues with no alternative oxidase protein had no alternative oxidase mRNA, suggesting that regulation occurred at the transcriptional level. During *S. guttatum* influorescence development and flowering, a dramatic increase of alternative oxidase activity occurs specifically in floral tissues (Elthon *et al.*, 1989). Accumulation of alternative oxidase protein and mRNA correlated with this developmentally timed, tissue specific increase in activity, suggesting that regulation occurs at the level of the transcript (Rhoads and McIntosh, 1992). However, *in vitro* transcription of isolated nuclei revealed no change in the rate of alternative oxidase transcription during this time period, indicating that accumulation of the transcript may be the controlling factor.

Treatment of suspension cells of *N. tabacum* with antimycin A, citrate, acetate, hydrogen peroxide, cysteine, or salicylic acid (Vanlerberghe and McIntosh, 1994; Vanlerberghe and McIntosh, 1996; Maxwell *et al.*, 2002) caused an increase in the steady state levels of alternative oxidase mRNA. This increase in transcript correlated with an increase in alternative oxidase protein (Vanlerberghe and McIntosh, 1992; Rhoads and McIntosh, 1993a; Vanlerberghe and McIntosh, 1996) and/or an increase in the capacity of the alternative pathway (Vanlerberghe and McIntosh, 1996; Maxwell *et al.*, 2002). Exposure of tobacco plants to drought, heat shock, cold, or high salt also caused an increase in the amount of alternative oxidase mRNA (Rizhsky *et al.*, 2002). Addition of antimycin A (Saisho *et al.*, 1997; Saisho *et al.*, 2001), or nitric oxide (Huang *et al.*, 2002) to *A. thaliana* cultures increased both alternative oxidase mRNA and capacity, and infection of leaves with *Pseudomonas syringae* (Simons *et al.*, 1999) caused an increase in alternative oxidase mRNA and protein. In *G. max*, alternative oxidase mRNA levels and protein increased in response to treatment with antimycin A, salicylic acid, citrate, or exposure to chilling (Djajanegara *et al.*, 2002). Thus, it is clear that a variety of stresses result in an increase in the amount of alternative oxidase transcript. It is unknown if the increase in amount of mRNA seen in these different plants is due to increased transcription of the alternative oxidase gene, an increased stability of the alternative oxidase transcript, or both.

In fungi and trypanosomes, the level of alternative oxidase activity and protein is also related to the amount of alternative oxidase transcript (Sakajo *et al.*, 1991; Li *et al.*, 1996; Yukioka *et al.*, 1998a; Yukioka *et al.*, 1998b; Joseph-Horne *et al.*, 1998; Borghouts *et al.*, 2001; Milani *et al.*, 2001; Huh and Kang, 2001; Chaudhuri *et al.*, 2002; Tanton *et al.*, 2003). Alternative oxidase activity, protein, and mRNA were seen constitutively in the fungi *G. graminis* (Joseph-Horne *et al.*, 1998) and *C. parapsilosis* (Milani *et al.*, 2001). The fungus *C. albicans* resembles several plant species in that it has two alternative oxidase genes, *AOX1a* and *AOX1b*, that seem to be regulated differentially (Huh and Kang, 2001). Addition of antimycin A, cyanide or hydrogen peroxide caused a substantial increase in *AOX1b* mRNA and alternative oxidase protein. Although *AOX1a* mRNA was not detectable by Northern blotting, reporter expression driven by the *AOX1a* promoter was constitutive. Low levels of *AOX1a* transcription may serve some housekeeping role in this fungus. Northern analyses of *P. anomala* (Sakajo *et al.*, 1991), *N. crassa* (Li *et al.*, 1996), *M. grisea* (Yukioka *et al.*, 1998b), and *P. anserina* (Borghouts *et al.*, 2001) showed that induction of alternative oxidase activity by inhibition of the cytochrome pathway correlated with an increase in alternative oxidase mRNA and protein. This correlation suggested that alternative oxidase was regulated transcriptionally or post-transcriptionally. Nuclear run-on assays in *M. grisea* (Yukioka *et al.*, 1998b), *T. brucei* (Chaudhuri *et al.*, 2002), and *N. crassa* (Tanton *et al.*, 2003) revealed that the alternative oxidase gene was constitutively transcribed at a low basal level even when protein and activity were not present. Induction of alternative oxidase by hydrogen peroxide or the fungicide (*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl)acetamide (SSF-126) in *M. grisea* (Yukioka *et al.*, 1998b), or by antimycin A in *N. crassa* (Tanton *et al.*, 2003), caused an increase in the transcription rate of the alternative oxidase gene. Treatment of *M. grisea* (Yukioka *et al.*, 1998b) or *T. brucei* (Chaudhuri *et al.*, 2002) with cycloheximide caused an increase in the amount of alternative oxidase transcript, suggesting that normally this mRNA is actively degraded by a *de novo* synthesized degradation factor. Expression of alternative oxidase may therefore be controlled at both transcriptional and post-transcriptional levels, altering the rates of both mRNA synthesis and degradation to adapt to respiratory needs.

1.8 Physiological role of alternative oxidase

Alternative oxidase is energetically wasteful (Moore *et al.*, 1978). Expression of the *S. guttatum* alternative oxidase in *S. pombe* resulted in reduced growth rate and fewer cells per mL at stationary phase, showing that the loss of energy from electron flow through alternative oxidase was physiologically significant (Affourtit *et al.*, 1999). Yet the persistence of this pathway in plants, fungi, algae and trypanosomes implies that it must have a significant beneficial function that outweighs the cost in energy.

The clearest known role for alternative oxidase is during thermogenesis in plant inflorescence development (James and Beevers, 1950; Bahr and Bonner, 1973; Meeuse, 1975). Alternative oxidase bypasses complexes III and IV, resulting in the release of potential energy as heat. Combining the alternative pathway with other mechanisms to increase heat production, such as increased overall respiration, decreased cytochrome capacity, uncouplers, and ATPases, results in the volatilization of aromatic compounds to attract pollinating insects.

For fungi, the physiological role of the alternative pathway is likely protective, as many plants release cyanide or nitric oxide to the environment, especially during the hypersensitivity response to fungal infection (Lambowitz and Zannoni, 1978; Vanlerberghe and McIntosh, 1997; Joseph-Horne *et al.*, 2001; Huang *et al.*, 2002; Veiga *et al.*, 2003a). The alternative pathway allows these organisms to survive despite inhibition of the normal cytochrome pathway (Lambowitz *et al.*, 1972b).

Alternative respiration was also suggested to accommodate surplus electron flow that cannot be used by a fully engaged cytochrome pathway (Lambowitz *et al.*, 1972b). Although flow through the alternative pathway results in less energy production, it allows the TCA cycle to continue, facilitating the biosynthesis of metabolites, and it drains excess reducing equivalents that could otherwise build up in the cell (Lambowitz *et al.*, 1972b; Meeuse, 1975; Palmer, 1976; Lambers, 1980; Lambers, 1982; Moore and Siedow, 1991; McIntosh, 1994; Moore *et al.*, 1994; Day *et al.*, 1995; Wagner and Krab, 1995; Pystina and Danilov, 2001; Veiga *et al.*, 2003b). This overflow function was suggested to occur in plants when there are excess sugars present, such as in young plants that have not yet developed a storage organ, during times of increased photosynthesis, or when sugars are not needed to maintain osmolarity (Lambers, 1980; Lambers, 1982; Vanlerberghe and McIntosh, 1997; Sluse and Jarmuszkiewicz, 1998).

An ancestral four helix bundle di-iron protein has been hypothesized to have been involved in ancient oxygen defenses based on the oxygen reducing capabilities of many of the members of the di-iron binuclear family of proteins (Gomes *et al.*, 2001), and modern day alternative oxidase may continue to play this important role. When the cytochrome pathway remains highly reduced, reduced forms of ubiquinol and complex I accumulate, a situation that can result in the production of damaging ROS (Joseph-Horne *et al.*, 2001). The availability of an alternative respiratory pathway can prevent this accumulation, and so may provide a protective effect against ROS damage (Whelan *et al.*, 1993; Day *et al.*, 1995; Wagner and Krab, 1995; Popov *et al.*, 1997). This hypothesized role for alternative oxidase is supported by a number of observations. Treatment of isolated mitochondria from *G. max* and *Pisum sativum* with the alternative oxidase inhibitors SHAM or propyl gallate caused an increase in the concentration of hydrogen peroxide (Popov *et al.*, 1997). Transgenic *N. tabacum* plants carrying alternative oxidase sense or antisense constructs have been used to increase or decrease alternative respiration, respectively (Vanlerberghe *et al.*, 1994). Non-invasive observation of ROS generation in mitochondria from these transgenic plants showed that plants carrying the sense construct had decreased levels of ROS, while antisense plants had increased levels (Maxwell *et al.*, 1999). The sense plants also had decreased levels of the ROS scavengers SOD and glutathione peroxidase; the antisense plants had increased levels of catalase and a pathogenesis related (PR) protein.

Treatments that affect cellular ROS levels also affect alternative oxidase activity. *N. tabacum* suspension cells grown in limited phosphate induced alternative oxidase, possibly to prevent ROS formation during the high energy production phase required to uptake phosphate (Parsons *et al.*, 1999; Yip and Vanlerberghe, 2001). Alternative oxidase was also induced by anoxia to decrease ROS production that occurs when oxygen returns. Pretreatment with anoxia or antimycin A protected *G. max* cells from hydrogen peroxide induced cell death, a protection that was abolished by SHAM or propyl gallate (Amor *et al.*, 2000). The compound historically known as "calorigen," responsible for provoking thermogenesis in *S. guttatum*, was identified to be salicylic acid (Raskin *et al.*, 1987). Salicylic acid increased alternative oxidase mRNA and protein in *P. hybrida*, *S. guttatum* and *N. tabacum* (Wagner, 1995; Rhoads and McIntosh, 1992; Rhoads and McIntosh, 1993a; Rhoads and McIntosh, 1993b), possibly because of its ability to bind and inhibit catalase and cause a rise in cellular hydrogen peroxide (Chen *et al.*, 1993). Chilling also induced alternative oxidase (McCaig and Hill, 1977; Yoshida and Tagawa, 1979; Elthon *et al.*, 1986; McNulty and Cummins, 1987; Vanlerberghe and McIntosh, 1992; Ito *et al.*, 1997; Gonzàlez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000; Abe *et al.*, 2002). The heat generated by the alternative pathway was not high enough to increase the temperature of the entire plant, but was suggested to generate enough heat to keep the MIM in a fluid state (Breidenbach *et al.*, 1997). Acclimation to lower temperatures by *Zea mays* resulted in increased hydrogen peroxide levels and expression of catalase (Prasad *et al.*, 1994), suggesting that alternative oxidase induction by chilling occurred through raised ROS levels.

The same protective role for alternative oxidase is seen in non-plant species. Hydrogen peroxide has been shown to induce alternative oxidase in *M. grisea* (Yukioka *et al.*, 1998b), *Acremonium chrysogenum* (Karaffa *et al.*, 2001), *P. anserina* (Borghouts *et al.*, 2001), *C. albicans* (Huh and Kang, 2001) and *H. capsulatum* (Johnson *et al.*, 2003), while treatment with ROS scavengers inhibited the induction of alternative oxidase in *P. anomala* (Minagawa *et al.*, 1992), *Pyricularia grisea* (Mizutani *et al.*, 1996), and *A. chrysogenum* (Karaffa *et al.*, 2001). Inhibition of alternative oxidase by SHAM increased ROS production and the activity of the ROS scavenger SOD in *T. brucei* (Fang and Beattie, 2003). In *C. albicans*, disruption or overexpression of the MnSOD resulted in an increase or decrease in alternative oxidase activity, respectively (Hwang *et al.*, 2003).

Another suggested function for alternative oxidase is as a biochemical pH-stat (Sakano, 1998). Plant cells use a proton gradient across the plasma membrane to facilitate secondary transport of ions, sugars, nucleotides, amino acids, and peptides (Palmgren, 2001). During times of environmental stress or high levels of cellular transport, the cytoplasm can become acidified. Along with secondary glycolysis pathways and fermentation, alternative oxidase could reduce the intracellular pH by the incorporation of hydrogen ions during the reduction of oxygen to form water (Sakano, 1998).

A number of observations also implicate a role for alternative oxidase in senescence. For example, when cultures of *N. tabacum* cells reached stationary phase, alternative oxidase was highly active (Rhoads and McIntosh, 1993a). Fruit ripening that is characterized by a peak in respiration referred to as climacteric. During the climacteric stage in *M. indica*, the cytochrome pathway was believed to provide enough energy for the respiratory burst, while alternative oxidase was involved in post-climacteric senescence (Considine *et al.*, 2001). Differential display of cultured *N. tabacum* cells showed that antimycin A, hydrogen peroxide and salicylic acid induced alternative oxidase mRNA along with transcripts known to be induced during senescence (Maxwell *et al.*, 2002). Addition of bongkrekic acid, an inhibitor of apoptotic PT pore formation, prevented alternative oxidase induction, which suggested that alternative oxidase was linked to programmed cell death. Addition of cysteine or antimycin A to *N. tabacum* cells expressing antisense alternative oxidase mRNA caused programmed cell death, with accumulation of typical DNA ladder-like fragments (Vanlerberghe *et al.*, 2002). These antisense plants also showed increased susceptibility to hydrogen peroxide, salicylic acid and cantharidin induction of cell death (Robson and Vanlerberghe, 2002). Increased alternative oxidase protein was seen during starvation induced aggregation of *Dictyostelium discoideum*, a stage at which cell death occurs (Jarmuszkiewicz *et al.*, 2002). Seedling cotyledons of *G. max* had a peak of alternative oxidase protein 20 days after imbibition, a developmental stage that involves senescence (Daley *et al.*, 2003). Senescence also occurs in *P. anserina*, and induction of alternative oxidase in a complex IV deficient background increased strain longevity (Dufour *et al.*, 2000; Borghouts *et al.*, 2001). However, over-expression or disruption of the alternative oxidase gene in cells with a wild-type oxidative phosphorylation system had no effect on life span (Lorin *et al.*, 2001). Alternative oxidase is evidently involved in senescence and programmed cell death, but whether this relationship is causative or indirect remains to be seen.

1.9 Alternative oxidase in *N. crassa*

Wild-type strains of *N. crassa* grown under standard lab conditions have cyanide-sensitive respiration. Cyanide-insensitive respiration was first seen in the cytoplasmically inherited [*poky*] mutant strains, which contained reduced levels of cytochromes *aa*$_3$ and *b*, and high levels of cytochrome *c* (Tissieres *et al.*, 1953; Lambowitz *et al.*, 1972a). The cyanide-insensitive respiratory pathway was shown to be sensitive to SHAM, suggesting similarity to the alternative respiration of plants (Lambowitz and Slayman, 1971). Growth of wild-type strains in the presence of antimycin A or cyanide, which inhibit electron flow through complex III or complex IV, respectively (Fig. 1), induced the alternative pathway (Lambowitz and Slayman, 1971). Growth in the presence of chloramphenicol, which inhibits mitochondrial translation (Schlünzen *et al.*, 2001) and leads to deficiencies in complex I, III and IV, also induced alternative respiration (Lambowitz and Slayman, 1971). A similar induction was seen when wild-type cells were grown in copper deficient media, which prevents the formation of cytochrome *aa*$_3$ (Schwab, 1973). The ability of chloramphenicol to induce alternative respiration suggested that both the regulatory pathway responsible for induction and the alternative pathway itself did not require translation of mtDNA encoded protein(s) (Lambowitz and Slayman, 1971). In mitochondria from [*poky*] or chloramphenicol induced wild-type strains, electron flow through the cytochrome pathway was near maximal while the alternative pathway was used for excess electrons (Lambowitz *et al.*, 1972b). The alternative pathway was shown to be induced within thirty minutes of the addition of chloramphenicol, at a time when the electron flow through the cytochrome pathway was predicted to be reduced only 10-15% (Edwards *et al.*, 1974). Induction continued and the capacity of the pathway increased linearly over 2.5 hours. The induction was blocked by actinomycin D or cycloheximide, so *de novo* transcription and translation were required to establish alternative respiration.

There have been two attempts to isolate alternative oxidase deficient mutants of *N. crassa*. In the first, six mutant strains sensitive to antimycin A and chloramphenicol were selected following ultraviolet (UV) mutagenesis (Edwards *et al.*, 1976). These mutations segregated in a Mendelian fashion and had normal cytochrome pathways. Some of these mutations fell into two complementation groups: *alx-1* consisting of strains

ANT-1, -4 and -6, and *alx-2* consisting of ANT-3. A further mutagenic screen was done using *N'*-nitro-*N*-nitrosoguanidine, and 21 antimycin A sensitive mutants were selected (Bertrand *et al.*, 1983). Complementation analysis showed that 17 of these mutants fell into the same complementation group as *alx-1*, which was renamed *aod-1*. The other four mutants were placed in the *aod-2* complementation group. Later analysis revealed that two of the strains designated as *aod-2* mutants were actually *aod-1* mutants (Li *et al.*, 1996; Tanton, 2003). Genetic mapping placed *aod-1* and *aod-2* near the centromeres of linkage group IV and II, respectively (Bertrand *et al.*, 1983).

The occurrence of alternative respiration in *N. crassa* correlated with the presence of a specific polypeptide, and it was shown that 19 of the 22 *aod-1* mutants had this protein while the two *aod-2* mutants tested did not (Bertrand *et al.*, 1983). Although the direct involvement of this polypeptide with alternative respiration was not shown, the pattern of its presence and absence suggested that *aod-1* was a structural gene and *aod-2* a regulatory gene. Subsequently, the monoclonal antibody against *S. guttatum* alternative oxidase (Elthon *et al.*, 1989) was used to show that the appearance of proteins in *N. crassa* with an apparent molecular weight of 36.5 and 37 kDa correlated with alternative respiration (Lambowitz *et al.*, 1989).

To isolate the *N. crassa* alternative oxidase structural gene, degenerate PCR primers were designed for highly conserved regions of the proteins deduced from previously sequenced alternative oxidase genes (Li *et al.*, 1996). The PCR product was used to screen a cosmid genomic DNA (gDNA) library and the *N. crassa* alternative oxidase gene was identified. The *aod-1* gene had typical *N. crassa* TATA box, CAAT box, transcription start site, and polyadenylation site sequences. A consensus cAMP response element (CRE) palindrome was found 739 bases upstream of the transcription start site. A cDNA was cloned and comparison with the genomic sequence revealed that the gene contains two introns with consensus splice site sequences. A 362 amino acid protein was predicted to be encoded by the gene, with an estimated size of 41.4 kDa. The N-terminus contained a predicted mitochondrial targeting presequence, removal of which would generate a mature protein with an estimated size of 34.6 kDa. High stringency Southern blotting suggested that there was only one copy of the alternative oxidase gene in the *N. crassa* genome (but see below). Sequencing of the gene from five *aod-1* mutant strains showed missense or frameshift mutations, while the *aod-1* gene in an *aod-2* strain had no mutations. Northern blot analysis correlated the presence of *aod-1* mRNA with induction of alternative oxidase activity. The role of *aod-2* was hypothesized to be a regulator of *aod-1* transcription or of transcript stability. A fusion of 3.3 kbp of *aod-1* upstream sequence with a cDNA copy of the *aod-1* gene was properly regulated in response to growth under non-inducing conditions or in the presence of chloramphenicol, suggesting that the intronic sequences do not play a regulatory role (C. Nargang and F. Nargang, personal communication).

Comparison of the upstream and intronic sequences of *aod-1* with the alternative oxidase genes from *Gelasinospora spp.* and *Aspergillus nidulans* showed that the CRE was the only conserved sequence element (Tanton *et al.*, 2003). The *N. crassa* CRE was shown to bind a protein in gel retardation assays but deletion of the CRE had no effect on regulation of alternative oxidase gene expression or activity. An upstream region of 255 bp was shown to be sufficient for proper control of *aod-1* expression in response to growth in the presence of chloramphenicol. Nuclear run-on assays revealed that *aod-1* was transcribed constitutively at a low level, but respiration assays and Western analysis showed that the constitutively produced transcript was not translated into protein. These assays also showed that the transcription was increased in the presence of antimycin A. Therefore, *N. crassa* alternative oxidase is regulated at the transcriptional level, with increased transcription in the presence of inducers, and at a post-transcriptional level, possibly through the transcript stability or by controlling initiation of translation.

With the release of the *N. crassa* genome sequence (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003) a second alternative oxidase structural gene called *aod-3* was found. Northern analysis revealed that *aod-3* transcripts could not be detected in cells grown in the presence of chloramphenicol, suggesting that the gene is not expressed in this condition (Tanton *et al.*, 2003). This observation is supported by the fact that *aod-1* mutant strains grown under alternative oxidase inducing conditions contain no cyanide-insensitive respiration.

1.10 Mitochondrial – nuclear communication

Coordination of gene expression from the mitochondrial and nuclear genomes is required for proper mitochondrial biogenesis and function (Poyton and McEwen, 1996; Surpin and Chory, 1997; Garesse and Vallejo, 2001). This coordination is necessary during normal growth for maintaining mitochondria, but also during times of stress, when nuclear gene expression can be modified in response to mitochondrial status, as is the case for alternative oxidase expression in *N. crassa*. These two organelles are able to affect each other's gene expression, implying that some form of communication or signaling exists between the two. Research into the cross-talk that is suspected to occur has shown that two-component signaling systems, ROS, and retrograde regulation factors are all involved.

1.10.1 Two-component signaling pathways

E. coli contains an *arcA*, *arcB* two-component system that is responsible for aerobic respiratory control (arc) exerted over the expression of enzymes involved in glycolysis, the TCA cycle, and electron transport (Iuchi and Lin, 1988; Iuchi *et al.*, 1989; Iuchi *et al.*, 1990; Allen, 1993; Iuchi and Lin, 1995; Lynch and Lin, 1996; Chang and Stewart, 1998). The evolutionary relationship between bacteria and mitochondria make this signaling pathway an attractive candidate for intra-organellar communication. The membrane embedded sensor *arcB* autophosphorylates at a histidine residue, possibly in response to the redox state of a protein involved in energy generation, or to the status of the proton gradient across the plasma membrane. This phosphate is then passed on to an aspartate residue on the mobile regulator *arcA*, a DNA binding protein, which results in either positive or negative regulation of gene expression by *arcA*.

Only one two-component signaling system has been found in *S. cerevisiae* and it acts as an osmosensor (Wurgler-Murphy and Saito, 1997; Hohmann, 2002). The Sln1p protein is located in the plasma membrane and under normal conditions autophosphorylates at a histidine residue. This phosphate is then passed to an aspartate in Sln1p, and then to a histidine in the phosphorelay protein Ypd1p. The phosphate group is finally used to phosphorylate either Ssk1p, a negative regulator of the high osmolarity glycerol (HOG) mitogen activated protein kinase (MAPK) signaling pathway, or the transcription factor Skn1p, thought to be involved in regulating the response to low osmolarity. Hyperosmotic shock causes a transient loss of phosphorylation of the sensors Sln1p and Ypd1p, and the regulators Ssk1p and Skn1p, and leads to activation of the HOG MAPK pathway.

In *C. albicans*, three putative histidine kinases have been discovered but have not yet been shown to take part in a phospho-relay signal transduction pathway (Yamada-Okabe *et al.*, 1999). Mutation of one of these putative histidine kinases decreased the constitutive low level of cyanide-insensitive respiration and alternative oxidase protein present in the fungus, but did not affect the ability of antimycin A to induce high levels of protein and activity (Huh and Kang, 2001). Since *C. albicans* could contain a two-component system that affects alternative oxidase regulation, it is possible that *N. crassa* also contains two-component signaling systems that may or may not be involved in nuclear-mitochondrial communication.

1.10.2 Reactive oxygen species as signaling molecules

Intracellular ROS have been implicated as signaling molecules that affect protein modification and gene expression (Sen and Packer, 1996; Finkel, 1998; Hancock *et al.*, 2001; Turpaev, 2002). An increase in ROS levels can cause activation of various kinases involved in intracellular signaling and transcription factors. One example of ROS signaling involves the mammalian protein kinase ASK1, an activator of a MAPK signaling pathway (Turpaev, 2002). In human cell cultures, the reduced form of the ROS scavenger thioredoxin was shown to bind and inactivate ASK1. Treatments that increased levels of ROS resulted in the oxidation of thioredoxin, release and activation of ASK1, and subsequent initiation of the MAPK cascade involved in activating transcription factors. One of the kinases involved in this MAPK cascade, JNK, was shown to be inactivated when bound to the ROS scavenger glutathione-S transferase. However, increased concentrations of ROSs disrupted this association, allowing JNK to become active and phosphorylate a number of transcription factors. ROS have also been shown to trigger the degradation of the inhibitory protein IκB, allowing the transcription factor NF-κB to localize to the nucleus and become active (Sen and Packer, 1996; Hancock *et al.*, 2001; Turpaev, 2002). ROS have been implicated in regulating alternative oxidase (see sections 1.7.5 and 1.8), and could possibly do so by acting as signaling molecules.

1.10.3 Retrograde regulation

S. cerevisiae contains a communication pathway between mitochondria and the nucleus called retrograde regulation (Poyton and McEwen, 1996; Surpin and Chory, 1997). Yeast strains that have disrupted mitochondrial function due to ρ^0 mtDNA, mutations in nuclear encoded mitochondrial proteins, or treatment with antimycin A have increased levels of the transcript for the peroxisomal citrate synthase, *CIT2* (Liao *et al.*, 1991). The increased amount of Cit2p in peroxisomes is thought to compensate for reduced mitochondrial function by increasing flow through the glyoxylate cycle, which produces malate and oxaloacetate for gluconeogenesis that provides more sugars for energy production. An upstream activation site (UAS) specific for the retrograde response was identified upstream of *CIT2* (Liao and Butow, 1993) and has also been found in the promoters of the *D*-lactate dehydrogenase gene *DLD3* (Chelstowska *et al.*, 1999), the mitochondrial citrate synthase gene *CIT1*, the aconitase gene *ACO1*, and the isocitrate dehydrogenase genes *IDH1* and *IDH2* (Liu and Butow, 1999). By co-regulating a subset of glyoxylate and TCA cycle enzymes, the yeast insures a steady cellular supply of citrate and α-ketoglutarate, a precursor of glutamate (Liu and Butow, 1999). Citrate is an important intermediate for both energetic pathways, and can shuttle between peroxisomes and mitochondria as required. Glutamate is an essential amino acid and a precursor for synthesis of other amino acids and nucleotides.

Three protein components of retrograde regulation have been identified. *RTG1* and *RTG3* have homology to basic helix-loop-helix zipper (bHLH/Zip) transcription factors (Liao and Butow, 1993). The Rtg1p-Rtg3p heterodimer was able to bind the *CIT2* UAS and activate transcription (Rothermal *et al.*, 1995; Rothermal *et al.*, 1997; Jia *et al.*, 1997). *RTG2* was found to act upstream of Rtg1p and Rtg3p during retrograde regulation (Liao and Butow, 1993; Rothermal *et al.*, 1997; Jia *et al.*, 1997). Rtg3p was normally sequestered in the cytosol by Rtg1p, but when retrograde regulation was initiated, Rtg3p was dephosphorylated in an Rtg2p-dependent manner, and Rtg1p-Rtg3p heterodimers localized to the nucleus (Sekito *et al.*, 2000). It is of interest to determine if *N. crassa* contains a similar retrograde regulatory pathway.

1.10.4 More than one signaling pathway

There is evidence for regulation of alternative oxidase gene expression through multiple pathways. Incubation of *G. max* suspension cells in the presence of citrate or antimycin A caused an increase in Aox1 mRNA and protein (Djajanegara *et al.*, 2002). However, treatment with the protein kinase inhibitor staurosporine blocked Aox1 induction by citrate but not by antimycin A, suggesting the involvement of two different signaling pathways. In *Zea mays*, inhibition of different oxidative phosphorylation complexes by mtDNA mutations or chemical inhibition caused expression of different *aox* family members (Karpova *et al.*, 2002). A complex I mutation or treatment of seedlings with rotenone resulted in increased levels of *aox2* mRNA, while a complex IV mutation or treatment with cyanide or antimycin A gave increased levels of *aox3* mRNA. Plants with a mutation causing defective mitochondrial translation had increased amounts of both *aox2* and *aox3* mRNA. Thus, each mutation or treatment caused induction of a specific gene or genes, suggesting that specific regulatory pathways are used. If *G. max* and *Z. mays* both contain at least two signaling pathways to induce alternative oxidase gene expression in response to specific treatments or disruption of specific complexes, then it is possible that *N. crassa* also contains more than one signaling pathway to induce *aod-1* expression.

1.11 Tyrosinase

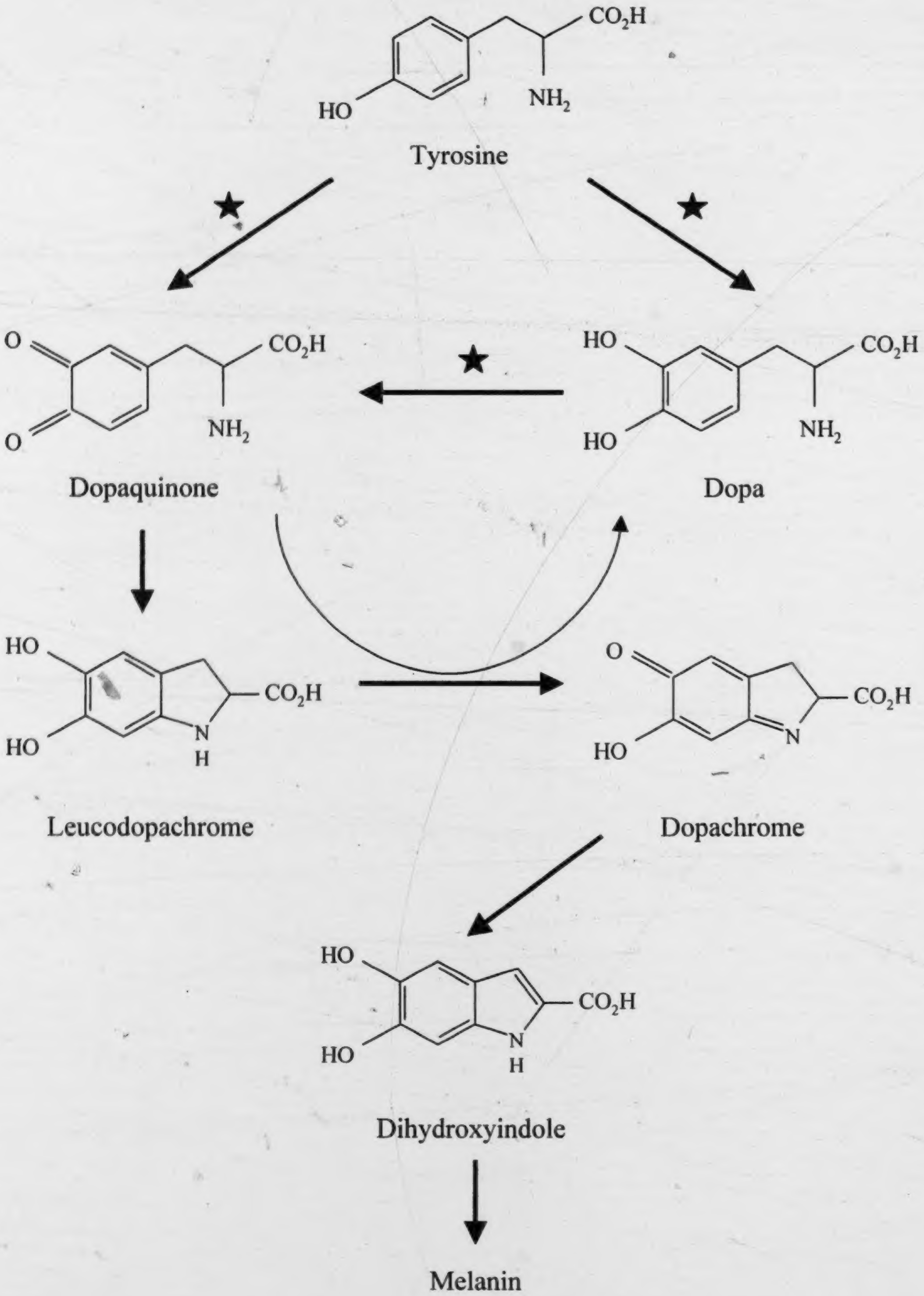
Previous efforts to identify genes involved in alternative oxidase regulation have used the lack of alternative oxidase activity itself as the basis of selection. This approach resulted in the isolation of 22 *aod-1* and 2 *aod-2* mutant strains, clearly indicating a bias towards mutation of the structural gene. In an attempt to avoid this predisposition, I chose to develop a reporter system using the enzyme tyrosinase that would exclude the isolation of *aod-1* structural gene mutations.

Past attempts to develop reporter systems in this laboratory were based on β-galactosidase (β-Gal), green fluorescent protein (GFP), and β-glucouronidase (GUS), and all were unsuccessful (Tanton and Nargang, personal communication). Recently, GFP has been expressed in *N. crassa* (Frietag *et al.*, 2001). It is believed that earlier attempts to use GFP failed because relatively weak promoters were used, and the original *Aequorea victoria* GFP sequence contained A-T rich codons, not suitable for *N. crassa*.

For the present work, the endogenous *N. crassa* enzyme tyrosinase, gene symbol *T*, was chosen as a potential reporter (Kothe *et al.*, 1993). In a previous search for strong *N. crassa* promoters, transformation of a fusion construct of the metallothionein promoter with *T* resulted in copper inducible tyrosinase expression and activity (Kupper *et al.*, 1990a). The tyrosinase reporter system was also used successfully to isolate two protein kinases, *nrc-1* and *nrc-2*, that affect expression of the gene *ccg-1*, an early regulator of entry into the developmental stage of conidiation (Kothe and Free, 1998). A brief review of the enzyme tyrosinase is given below.

Tyrosinase (monophenol monooxygenase EC 1.14.18.1) is an enzyme involved in melanogenesis, the process by which the pigment melanin is formed (reviewed in García-Borrón and Solano, 2002; Seo *et al.*, 2003; Langfelder *et al.*, 2003). The enzyme is able to catalyze the oxidation of both monophenols and *o*-diphenols to produce *o*-quinols. Tyrosinase has been found in both prokaryotes and eukaryotes, including plants, animals, fungi and microorganisms. In lower organisms, including *N. crassa*, tyrosinase is the only enzyme required for melanogenesis (Seo *et al.*, 2003; Langfelder *et al.*, 2003). Tyrosinase catalyzes the rate limiting initial reactions, while subsequent reactions occur non-enzymatically (Fig. 4). The active site of tyrosinase is predicted to form a four helix bundle in a hydrophobic pocket (García-Borrón and Solano, 2002; Seo *et al.*, 2003; Langfelder *et al.*, 2003). Two copper ions are each coordinated by three histidine residues to form the binuclear copper site that binds oxygen. The conserved active site has homology to hemocyanins, the copper containing oxygen carriers in the hemolymph of arthropods and molluscs.

Studies of tyrosinase in *N. crassa* began with the observation that thawed homogenates of cells or aging mycelia produced a brown pigment, which was identified as melanin (Fox and Gray, 1950). Wild-type cultures grown in standard media have little to no tyrosinase activity (Horowitz and Shen, 1952). During the sexual cycle the female of the cross produces perithecia, and both perithecia and ascospores contain melanin


Tyrosine
Dopaquinone
Dopa
Leucodopachrome
Dopachrome
Dihydroxyindole
Melanin

(Hirsch, 1954). As protoperithecia develop, they change from a pale colour to dark brown as melanin is made. Analysis of extracts reveals that tyrosinase activity peaks at the same time as the rate of protoperithecial formation peaks. The involvement of tyrosinase in the mating cycle could explain why tyrosinase mutant strains are female sterile (Fuentes *et al.*, 1994).

The amino acid sequence of the enzyme was determined by analysis of chemical cleavage products (Lerch, 1978; Lerch *et al.*, 1982). Mature tyrosinase was determined to be a 407 amino acid protein with a predicted weight of 46 kDa. In isolated mycelial extracts, tyrosinase activity was increased by overnight dialysis (Horowitz and Shen, 1952), incubation of the extract at cold temperatures, addition of sodium ions, urea, triethylaminesuccinate buffer, tris 2-amino-2-hydroxymethylpropane-1:3-diol (Gest and Horowitz, 1958), or addition of the original growth media back to the extract (Fox and Burnett, 1959). These observations led to the hypothesis that tyrosinase was synthesized in an inactive form. Sequencing of the tyrosinase gene revealed an encoded C-terminal extension that was not present in the mature purified protein (Kupper *et al.*, 1989). Cleavage of this domain was followed using polyclonal antibodies generated against purified mature tyrosinase or against a fusion protein of GST and the C-terminal domain (Kupper *et al.*, 1990b). Western analysis showed three bands with apparent molecular weights of 75 kDa, 52 kDa, and 46 kDa, which they hypothesized correlated with protyrosinase, an intermediate form, and mature tyrosinase, respectively. The C-terminal specific antibody failed to recognize the mature tyrosinase species, suggesting that cleavage of the C-terminal extension occurred during processing of the intermediate to the mature form. Southern and Western analyses showed that other *Neurospora* species *N. intermedia*, *N. sitophila*, *N. africanus*, and *N. dodgei*, but not *N. tetrasperma* also encode a tyrosinase with a C-terminal extension (Kupper *et al.*, 1989). Hemocyanin from the lobster *Panulirus interruptus* has a C-terminal domain that interacts with and possibly shields the active site. It was suggested that the C-terminal extension of *N. crassa* tyrosinase could interact with the active site and must be removed for activity (Kupper *et al.*, 1989). The fungal enzyme is also post-translationally modified with N-acetylation at the N-terminal serine residue and formation of a histidylcysteine thioether bridge between amino acids 94 and 96 (Lerch *et al.*, 1982).

The *T* gene was cloned using degenerate primers based on the known amino acid sequence (Kupper *et al.*, 1989). The *T* gene is 2016 base pairs in length, and contains two introns at positions +151 and +1068, with lengths of 52 and 99 base pairs, respectively. The length of the gene and the derived amino acid sequence confirmed that tyrosinase is synthesized as a precursor with a C-terminal extension. Maturation of the enzyme requires cleavage after amino acid 407, removing the C-terminal 213 amino acids. A typical transcription start site, TATA box and intron consensus sequences for *N. crassa* are present, and the gene is also typically G-C rich. The promoter region was scanned for known regulatory elements and a sequence differing by one base from a cAMP response element (CRE) palindrome was found at position -124 (Kupper *et al.*, 1989). It was suggested that the gene is regulated by the CRE at the transcriptional level, and that the presence of a CRE may be responsible for the suggested catabolite repression of tyrosinase (Feldman and Thayer, 1974). Northern analysis shows that regulation of tyrosinase activity by cAMP is at the transcriptional or post-transcriptional level, either by increasing mRNA synthesis or stability (Kupper *et al.*, 1990b). Tyrosinase mRNA is present fifteen minutes after induction by cAMP and the mRNA level peaks at twenty-four hours. Tyrosinase activity appears two hours after cAMP induction and peaks at forty-eight hours.

1.12 Objective of this study

The major objective of this thesis was to identify genes involved in alternative oxidase regulation in *N. crassa*. An understanding of this regulation may serve as a model for how mitochondria are able to induce gene expression in the nucleus. *N. crassa* is a good model organism for this investigation, since it contains a well characterized alternative respiratory pathway and is relatively easily used for genetic analysis. Prior to this study, no gene from any species had been implicated in playing a regulatory role with respect to alternative oxidase except *aod-2*, and the identity and function of this gene product has remained elusive. Mutagenesis of the reporter carrying strain of *N. crassa*, and analysis of the derived mutant strains has led to the isolation of four novel alternative oxidase regulatory genes. The study of these mutations could provide insight not only into this specific regulatory pathway, but potentially into intracellular communication.

2 Materials and Methods

2.1 Growth of *N. crassa*

Strains of *N. crassa* used are listed in Tables 1 to 3, and were grown and handled as described by Davis and de Serres (1970). Vegetative growth was on standard Vogel's media with supplements as necessary, and solid media contained 1.6% agar. To promote growth into colonies, conidia were spread on media where 1.5% sucrose was replaced with 1.5% sorbose, 0.05% glucose, and 0.05% fructose. Slants with conidia were placed at –80°C for long term storage.

To obtain large amounts of conidia to inoculate liquid cultures, a small inoculum from the strain of interest was placed in a conidia flask, a 250 mL Erlenmeyer flask containing 50 mL of solid media. These flasks were typically incubated at 30°C for two days, but longer if mycelia had not yet spread up the flask walls. Once mycelia had grown sufficiently, flasks were placed in the light at room temperature for about seven days to promote conidiation. If lesser amounts of conidia were required, conidia were inoculated onto a slant. Similar growth conditions for slants were used as described above for conidia flasks. To harvest conidia, sterile pre-chilled distilled H_2O (dH_2O) was placed in the flask or slant and swirled to suspend the conidia. Conidial suspensions from flasks were passed through a cheesecloth filter to remove mycelia.

Liquid cultures were grown overnight (14 to18 hr) at 30°C. Cultures of 50 to 500 mL were grown in Erlenmeyer flasks with shaking at 150 revolutions per minute (rpm) for aeration. The total volume of the flasks was at least four times the volume of media used, and the flasks were baffled for volumes larger than 50 mL to insure adequate aeration of the culture. Larger cultures were grown in "bubblers," where filled 2 L flasks were placed in a 30°C water bath and aerated by bubbling filtered air into the flask using sterile tubing. Strains were inoculated at 1×10^6 conidia/mL unless large numbers of small cultures were being inoculated, in which case the amount of inoculum was estimated. Inoculation with 0.5 mL of a solution obtained from harvesting a single large slant (18 x 150 mm tube with 10 mL media) with 8 mL of sterile dH_2O usually gave an inoculation of approximately 1×10^6 conidia/mL. This estimation was used for large numbers of respiration assays only. Strains were often grown in the presence of

Table 1.

Strains used during this study.

Strain	Source	Genotype
NCN5	Nargang Lab	*cya-5, A*
NCN10	A. Lambowitz	*nic-1, al-2, A*
NCN233	Nargang Lab	*pan-2, A*
NCN235	Nargang Lab	*pan-2, a*
NCN246	Nargang Lab	*pyr-6, A*
NCN251-5	Nargang Lab	*A*
nuc-2 *A*	R.L. Metzenberg	*nuc-2, A*
nuc-2 *a*	R.L. Metzenberg	*nuc-2, a*
763	FGSC[1]	*nic-1, A*
764	FGSC	*nic-1, a*
810	FGSC	*os-1, a*
997	FGSC	*alcoy, A*
998	FGSC	*alcoy, a*
1205	FGSC	*al-1, arg-5, A*
1206	FGSC	*al-1, arg-5, a*
1208	FGSC	*trp-1, ylo, a*
1243	FGSC	*inl, cot-1, A*
1244	FGSC	*inl, cot-1, a*
2283	FGSC	*un-5, al-2, arg-13, a*
2997	FGSC	*pyr-4, arg-12, A*
2998	FGSC	*pyr-4, arg-12, a*
3752	FGSC	*caf-1, al-3, his-6, A*
3753	FGSC	*caf-1, al-3, his-6, a*
3789	FGSC	*ro-10, al-2, un-18, A*
3790	FGSC	*ro-10, al-2, un-18, a*
4006	FGSC	*nic-2, A*
4007	FGSC	*nic-2, a*
4009	FGSC	*cr-1, a*
4065	FGSC	*cot-1, A*
4119	FGSC	*acr-2, trp-1, dow, A*
4120	FGSC	*acr-2, trp-1, dow, a*
4121	FGSC	*cys-10, pdx-1, pan-1, A*
4122	FGSC	*cys-10, pdx-1, pan-1, a*
4141	FGSC	*nic-3, met-7, arg-10, A*
4142	FGSC	*nic-3, met-7, arg-10, a*
4451	FGSC	*thi-4, nic-3, ars-1, a*
4471	FGSC	*nic-3, ars-1, A*
5138	FGSC	*cpk, A*

Table 1 continued

Strain	Source	Genotype
6563	FGSC	*ro-7, arg-5, rip-1, A*
6564	FGSC	*ro-7, arg-5, rip-1, a*
6808	FGSC	*trp-1, ylo-1, A*
7094	FGSC	*mcb, a*
7224	FGSC	*ro-10, nit-2, leu-3, A*
7225	FGSC	*ro-10, nit-2, leu-3, a*
7255	FGSC	*cyh-2, lys-2, leu-2, mei-2, a*
7258	FGSC	*pyr-4, inl, inv, mei-2, a*
7261	FGSC	*helper*[2] *+ ad-3A, am132, inl, inv, mei-2, A*
7262	FGSC	*helper + pyr-3, trp-3, am132, inl, inv, mei-2, A*
7263	FGSC	*helper + ad-2, am132, inl, inv, mei-2, A*
7264	FGSC	*helper + trp-4, am132, inl, inv, mei-2, A*
7265	FGSC	*helper + am132, inl, inv, mei-2, A*
7266	FGSC	*helper + ad-1, am132, inl, inv, mei-2, A*
7267	FGSC	*helper + met-7, am132, inl, inv, mei-2, A*
7355	FGSC	*cot-5, het-COR, pyr-4, thr-2, A*
7356	FGSC	*cot-5, het-COR, pyr-4, thr-2, a*
7563	FGSC	*nit-6, ylo-1, un-23, A*
7564	FGSC	*nit-6, ylo-1, un-23, a*
T1P11	S.J. Free	*T, al-2, a*
T1P11P08	S.J. Free	*T, al-2, a*
T1P11P13	S.J. Free	*T, al-2*
T1P11P16	S.J. Free	*T, al-2*
8-18	This study	*T, al-2, a* + pBAT[3]
T11-76	This study	*T, al-2, a* + pBAT
7207	H. Bertrand	*aod-1, pan-2, A*
AIN-19	This study	*aod-1, nic-3, A*
AA1-15	I. Cleary	*aod-1, arg-5, A*
AA1-27, -37	I. Cleary	*aod-1, al-1, arg-5, A*
AA1-42	I. Cleary	*aod-1, al-1, arg-5, a*
7064	H. Bertrand	*aod-2-4, nic-1, al-2, a*

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[1] FGSC is the Fungal Genetics Stock Center at the University of Kansas Medical Center, Kansas City.

[2] Strains with the designation "helper +" are maintained as heterokaryons with a sterile helper strain.

[3] The indication "+ pBAT" means that the strain carries an integrated copy of the pBAT plasmid.

Table 2.

Isolated mutant strains and their progeny.

Strain	Origin	Genotype	Gene Designation[1]
2-195		*E1*[2]*, T, al-2, a* + pBAT[3]	*aod-4*
EN195-26	NCN233 X 2-195[4]	*E1, pan-2, al-2, a,* + pBAT	*aod-4*
EN195-34	NCN233 X 2-195	*E1, pan-2, al-2, A,* + pBAT	*aod-4*
EN195-44	NCN233 X 2-195	*E1, pan-2, al-2, a,* + pBAT	*aod-4*
EN195-109	NCN233 X 2-195	*E1, pan-2, a*	*aod-4*
2E1a-57	EN195-109 X 1243	*E1, inl, cot-1, A*	*aod-4*
4-294		*E2, T, al-2, a* + pBAT	*chl-1*
EN294-35	NCN246 X 4-294	*E2, pyr-6, al-2, a*	*chl-1*
EN294-40	NCN246 X 4-294	*E2, pyr-6, A* + pBAT	*chl-1*
EN294-46	NCN246 X 4-294	*E2, pyr-6, A*	*chl-1*
E2a-119	1206 X EN294-46	*E2, arg-5, pyr-6, A*	*chl-1*
5-14		*E3, T, al-2, a* + pBAT	*aod-2*
EN14-1	7263 X 5-14	*E3, ad-2, al-2, A* + pBAT	*aod-2*
EN14-2	7263 X 5-14	*E3, ad-2, a* + pBAT	*aod-2*
EN14-10	7263 X 5-14	*E3, ad-2, a* + pBAT	*aod-2*
EN14-18	7263 X 5-14	*E3, ad-2, al-2, a* + pBAT	*aod-2*
EN14-22	7263 X 5-14	*E3, ad-2, al-2, A* + pBAT	*aod-2*
EN14-25	7263 X 5-14	*E3, ad-2, A* + pBAT	*aod-2*
EN14-34	7263 X 5-14	*E3, ad-2, al-2, A* + pBAT	*aod-2*
EN14-36	7263 X 5-14	*E3, ad-2, a* + pBAT	*aod-2*
EN14-158	7263 X 5-14	*E3, ad-2, A*	*aod-2*
5-34		*E4, T, al-2, a* + pBAT	
L1-6		*E5, T, al-2, a* + pBAT	
L1-13		*E6, T, al-2, a* + pBAT	
6-280		*E7, T, al-2, a* + pBAT	
L2-25		*E8, T, al-2, a* + pBAT	*aod-4*
EL25-6	7267 X L2-25	*E8, met-7, al-2, A* + pBAT	*aod-4*
EL25-27	7267 X L2-25	*E8, met-7, a* + pBAT	*aod-4*
EL25-37	7267 X L2-25	*E8, met-7, A* + pBAT	*aod-4*
L2-37		*E9, T, al-2, a* + pBAT	*aod-4*
L2-40		*E10, T, al-2, a* + pBAT	*aod-5*
L2-61		*E11, T, al-2, a* + pBAT	*aod-4*
NL61-130	763 X L2-61	*E11, nic-1, A*	*aod-4*
E11a-40	NL61-130 X 1244	*E11, inl, A*	*aod-4*
L2-62		*E12, T, al-2, a* + pBAT	*aod-6*
EL62-2	7262 X L2-62	*E12, trp-4, A*	*aod-6*
EL62-25	7262 X L2-62	*E12, trp-4, A*	*aod-6*
L2-64		*E13, T, al-2, a* + pBAT	*aod-4*

Table 2 continued

Strain	Origin	Genotype	Gene Designation
EL64-46	7266 X L2-64	*E13, ad-1, al-2, a* + pBAT	*aod-4*
EL64-47	7266 X L2-64	*E13, ad-1, A* + pBAT	*aod-4*
L2-67		*E14, T, al-2, a* + pBAT	*aod-4*
EL67-2	7261 X L2-67	*E14, ad-3A, al-2, A* + pBAT	*aod-4*
EL67-6	7261 X L2-67	*E14, ad-3A, A* + pBAT	*aod-4*
EL67-33	7261 X L2-67	*E14, ad-3A, A* + pBAT	*aod-4*
EL67-46	7261 X L2-67	*E14, ad-3A, A* + pBAT	*aod-4*
7-64		*E15, T, al-2, a* + pBAT	*aod-7*
L3-4		*E16, T, al-2, a* + pBAT	
L3-5		*E17, T, al-2, a* + pBAT	
L3-8		*E18, T, al-2, a* + pBAT	

[1] The designations *aod-4*, *aod-5*, *aod-6*, *aod-7*, and *chl-1* are proposed in this thesis for the new mutants isolated as described in the results section.

[2] The designations *E1* to *E18* refer to the original isolation numbers assigned to the mutations.

[3] The indication "+ pBAT" means that the strain carries an integrated copy of the pBAT plasmid.

[4] The female of the cross is the strain listed first.

Table 3.

Heterokaryotic strains created for complementation analysis.

Heterokaryon	Mutations Tested for Complementation
EN195-26 + AA1-42	*E1, aod-1*
EN195-109 + AA1-42	*E1, aod-1*
EN195-34 + AA1-15	*E1, aod-1*
EN195-34 + AA1-27	*E1, aod-1*
EN195-34 + AA1-37	*E1, aod-1*
EN195-26 + 7064	*E1, aod-2*
EN195-44 + 7064	*E1, aod-2*
EN195-26 + EN294-35	*E1, E2*
EN195-44 + EN294-35	*E1, E2*
EN195-26 + EN14-2	*E1, E3*
EN195-26 + EN14-18	*E1, E3*
EN195-26 + EL25-27	*E1, E8*
EN195-34 + NL61-130	*E1, E11*
EN195-34 + EL62-2	*E1, E12*
EN195-34 + EL62-25	*E1, E12*
EN195-26 + EL64-46	*E1, E13*
EN195-34 + EL67-2	*E1, E14*
EN195-34 + EL67-6	*E1, E14*
EN195-34 + EL67-33	*E1, E14*
EN195-34 + EL67-46	*E1, E14*
EN294-40 + 7207	*E2, aod-1*
EN294-46 + 7207	*E2, aod-1*
EN294-35 + 7064	*E2, aod-2*
EN294-35 + EN14-2	*E2, E3*
EN294-35 + EN14-18	*E2, E3*
E2a-119 + EL25-6	*E2, E8*
E2a-119 + EL25-37	*E2, E8*
E2a-119 + NL61-130	*E2, E11*
EN294-46 + EL62-2	*E2, E12*
EN294-46 + EL62-25	*E2, E12*
EN294-46 + EL64-47	*E2, E13*
EN294-46 + EL67-2	*E2, E14*
EN294-46 + EL67-33	*E2, E14*
EN294-46 + EL67-46	*E2, E14*
EN14-22 + 7207	*E3, aod-1*
EN14-34 + 7207	*E3, aod-1*
EN14-158 + 7207	*E3, aod-1*
EN14-2 + AA1-42	*E3, aod-1*

Table 3 continued

Heterokaryon	Mutations Tested for Complementation
EN14-10 + AA1-42	*E3, aod-1*
EN14-36 + AA1-42	*E3, aod-1*
EN14-22 + AA1-15	*E3, aod-1*
EN14-22 + AA1-27	*E3, aod-1*
EN14-22 + AA1-37	*E3, aod-1*
EN14-25 + AA1-15	*E3, aod-1*
EN14-25 + AA1-27	*E3, aod-1*
EN14-25 + AA1-37	*E3, aod-1*
EN14-34 + AA1-15	*E3, aod-1*
EN14-34 + AA1-27	*E3, aod-1*
EN14-34 + AA1-37	*E3, aod-1*
EN14-158 + AA1-15	*E3, aod-1*
EN14-158 + AA1-27	*E3, aod-1*
EN14-158 + AA1-37	*E3, aod-1*
EN14-2 + 7064	*E3, aod-2*
EN14-18 + 7064	*E3, aod-2*
EN14-34 + NCN233	*E3*
EN14-34 + NCN246	*E3*
EN14-158 + EL25-6	*E3, E8*
EN14-158 + EL25-37	*E3, E8*
EN14-158 + NL61-130	*E3, E11*
EN14-34 + EL62-2	*E3, E12*
EN14-34 + EL62-25	*E3, E12*
EN14-34 + EL64-47	*E3, E13*
EN14-34 + EL67-2	*E3, E14*
EN14-34 + EL67-33	*E3, E14*
EN14-34 + EL67-46	*E3, E14*
EL25-6 + AA1-15	*E8, aod-1*
EL25-6 + AA1-27	*E8, aod-1*
EL25-6 + AA1-37	*E8, aod-1*
EL25-37 + AA1-15	*E8, aod-1*
EL25-37 + AA1-27	*E8, aod-1*
EL25-37 + AA1-37	*E8, aod-1*
EL25-27 + 7064	*E8, aod-2*
EL25-6 + NL61-130	*E8, E11*
EL25-37 + NL61-130	*E8, E11*
EL25-6 + EL62-2	*E8, E12*
EL25-6 + EL62-25	*E8, E12*
EL25-37 + EL62-2	*E8, E12*

Table 3 continued

Heterokaryon	Mutations Tested for Complementation
EL25-37 + EL62-25	*E8, E12*
EL25-27 + EL64-46	*E8, E13*
EL25-6 + EL67-2	*E8, E14*
EL25-6 + EL67-6	*E8, E14*
EL25-6 + EL67-33	*E8, E14*
EL25-6 + EL67-46	*E8, E14*
EL25-37 + EL67-2	*E8, E14*
EL25-37 + EL67-6	*E8, E14*
EL25-37 + EL67-33	*E8, E14*
EL25-37 + EL67-46	*E8, E14*
NL61-130 + 7207	*E11, aod-1*
NL61-130 + AA1-15	*E11, aod-1*
NL61-130 + AA1-27	*E11, aod-1*
NL61-130 + AA1-37	*E11, aod-1*
NL61-130 + CNA-33	*E11, aod-2*
NL61-130 + EL62-2	*E11, E12*
NL61-130 + EL62-25	*E11, E12*
NL61-130 + EL64-47	*E11, E13*
NL61-130 + EL67-2	*E11, E14*
NL61-130 + EL67-6	*E11, E14*
NL61-130 + EL67-33	*E11, E14*
NL61-130 + EL67-46	*E11, E14*
EL62-2 + 7207	*E12, aod-1*
EL62-25 + 7207	*E12, aod-1*
EL62-2 + CNA-33	*E12, aod-2*
EL62-25 + CNA-33	*E12, aod-2*
EL62-2 + EL64-47	*E12, E13*
EL62-25 + EL64-47	*E12, E13*
EL62-2 + EL67-2	*E12, E14*
EL62-2 + EL67-6	*E12, E14*
EL62-2 + EL67-33	*E12, E14*
EL62-2 + EL67-46	*E12, E14*
EL62-25 + EL67-2	*E12, E14*
EL62-25 + EL67-6	*E12, E14*
EL62-25 + EL67-33	*E12, E14*
EL62-25 + EL67-46	*E12, E14*
EL64-47 + 7207	*E13, aod-1*
EL64-46 + 7064	*E13, aod-2*
EL64-47 + EL67-2	*E13, E14*

Table 3 continued

Heterokaryon	**Mutations Tested for Complementation**
EL64-47 + EL67-6	*E13, E14*
EL64-47 + EL67-33	*E13, E14*
EL64-47 + EL67-46	*E13, E14*
EL67-2 + 7207	*E14, aod-1*
EL67-33 + 7207	*E14, aod-1*
EL67-46 + 7207	*E14, aod-1*
EL67-2 + CNA-33	*E14, aod-2*
EL67-6 + CNA-33	*E14, aod-2*
EL67-33 + CNA-33	*E14, aod-2*
EL67-46 + CNA-33	*E14, aod-2*

chloramphenicol (2 mg/mL), or antimycin A (0.5 μg/mL) to induce alternative oxidase activity.

2.2 Yeast one hybrid vector and transformation

A 31 bp fragment containing the 8 bp CRE had been amplified by PCR and four copies of this fragment had been cloned in tandem into the *Bam*HI site of pBluescript to generate plasmid pCH105 (Table 4; F. Nargang, unpublished results). The tandem copies were excised intact from pCH105 by digestion with *Sst*I and *Eco*RV. Plasmid pCHisi-1 was created by ligating the fragment containing the 4 CREs into the MATCHMAKER one hybrid vector pHisi-1 (Clontech, Palo Alto, CA), which had been digested with *Xba*I, blunt ended, digested with *Sst*I, and then dephosphorylated (Table 4).

This construct was transformed into yeast strain YM4271 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-Δ538, gal80- Δ538, ade5::hisG*). Yeast growth and transformation was performed as described by the manufacturers of the MATCHMAKER one hybrid kit (Clontech, Palo Alto, CA). Leaky expression from the *HIS3* gene was used to select transformants. Strain YM4271 was also transformed with a supplied control plasmid carrying 3 tandem copies of the p53 binding site upstream of the *HIS3* gene. A pCHisi-1 transformant strain was grown on media containing various concentrations of the His3p inhibitor 3-amino-1,2,4-triazole (3-AT), and its growth was compared to that of a strain transformed with the control plasmid.

2.3 Tyrosinase reporter

Tyrosinase mutant strains and plasmid pTYR103 containing the *T* gene were obtained from the lab of Stephen Free (Table 4; Kothe *et al.*, 1993; Fuentes *et al.*, 1994). A restriction fragment containing 18 bp of 5' sequence found upstream of the *T* translational start codon, the entire coding sequence for *T*, and 3' sequence including the polyadenylation signal had been cloned into pBluescript II SK$^-$ to generate pTYR103 (Kothe *et al.*, 1993). This plasmid has unique restriction sites for *Eco*RI and *Xba*I upstream of the tyrosinase sequences. The *T* mutant strain T1P11 was created by crossing a wild-type strain with a strain containing an ectopically integrated copy of pTYR103 to give a duplication of the *T* gene (S. Free, personal communication). The duplication

Table 4.
Plasmids used during this study.

Vector	Source	Description
pHisi-1	Clonetech Laboratories Inc.	Yeast integration vector, carries *HIS3* gene
pCH105	F. Nargang	Four copies of 31 bp fragment containing *aod-1* CRE cloned in pBluescript
pCHisi-1	This Study	Four tandem copies of CRE fragment cloned into pHisi-1 upstream of yeast *HIS3* gene
pTYR103	S. Free	Tyrosinase coding, 18 bp upstream and 3' UTR sequences cloned in pBluescript
pAOPFGuB	L. Tanton	3.3 kbp *aod-1* upstream sequence, β-glucouronidase gene, and bleomycin resistance gene cloned in pGUS (Clonetech Palo Alto, CA; L. Tanton, 2003)
pAT	This Study	3.3 kbp *aod-1* upstream sequence placed upstream of tyrosinase gene in pTYR103
pAB520	B. Austin *et al.*	Bleomycin resistance gene cloned in pUC18
pBAT	This Study	pBAT with bleomycin resistance gene from pAB520 cloned into *Xba* I site
pCSN43	C. Staben *et al.*	Hygromycin resistance gene cloned in pBluescript

served as a substrate for the process of repeat induced point mutation (RIP) during the premeiotic phase of the cross (Selker, 1990). RIP typically causes C to T and G to A transition mutations and methylation of duplicated sequences, resulting in loss of expression and/or function of the target gene. T1P11 inherited the RIPed copy of the endogenous *T* gene and did not inherit the ectopically integrated pTYR103 plasmid sequences. T1P11 was then backcrossed to a wild-type strain and progeny T1P11P08, T1P11P13 and T1P11P16 were isolated that contained the RIPed copy of *T* (S. Free, personal communication). All four strains should therefore contain the same RIP generated mutations (but see section 3.6).

2.4 Construction of reporter system

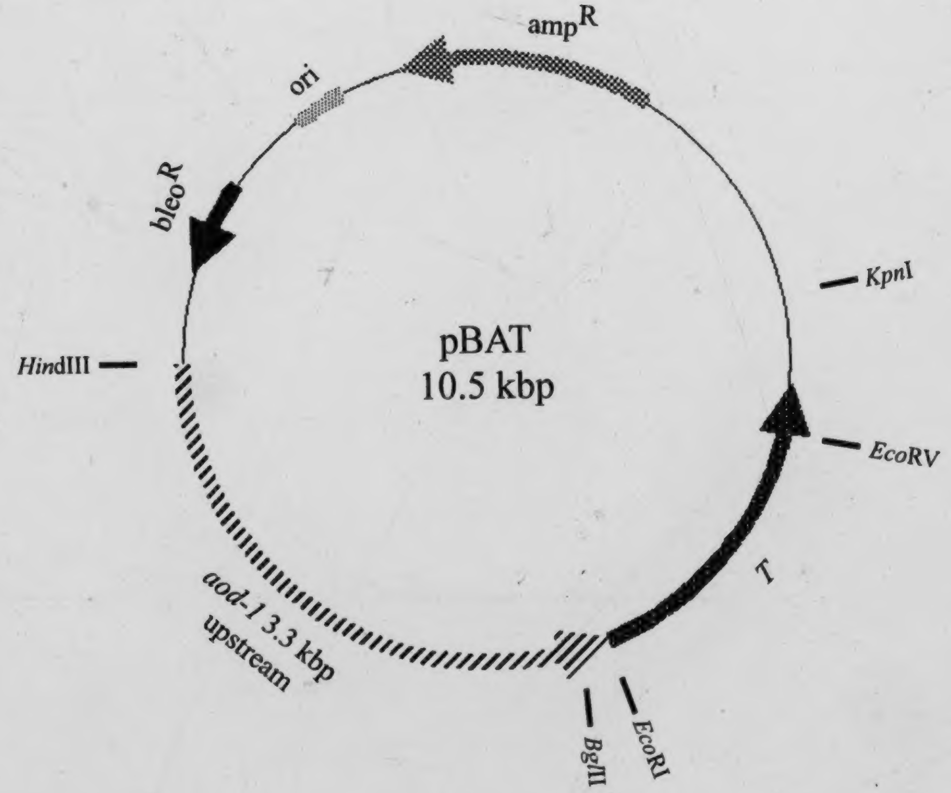
Digestion of the plasmid pAOPFGuB (Table 4; Tanton, 2003) with *Hin*dIII and *Spe*I liberated a 3347 bp fragment spanning –3304 to +43 (relative to the *aod-1* transcription start +1) of the alternative oxidase upstream region, which ended 13 bp before the AUG start codon of the alternative oxidase structural gene. This upstream fragment was blunt ended and cloned into the blunted *Eco*RI site of the vector pTYR103 (Kothe *et al.*, 1993), placing it upstream of the tyrosinase gene to yield plasmid pAT. A 1.6 kbp *Xba*I fragment from the plasmid pAB520 (Table 4; Austin *et al.*, 1990) containing the selectable marker for bleomycin resistance was cloned into the *Xba*I site of pAT, generating the reporter construct pBAT (Table 4; Fig. 5A). The fusion of the 3' end of the 3.347 kbp promoter fragment with the upstream sequence of the tyrosinase gene is shown (Fig. 5B). pBAT contains unique restriction sites for *Kpn*I, *Eco*RI, *Eco*RV, *Bgl*II, and *Hin*dIII.

2.5 DNA modification and gel electrophoresis

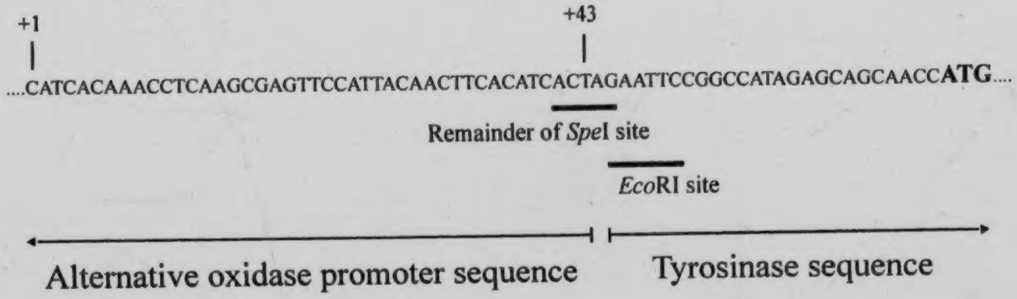
Restriction endonucleases, T4 DNA ligase, RNase A, Klenow fragment and DNA phosphatases used were purchased from Gibco BRL/Invitrogen (Burlington, ON), Amersham Pharmacia Biotech (San Francisco, CA), New England Biolabs (Mississauga, ON) or Roche Scientific (Laval, QB). Reactions were carried out according to manufacturer's directions.

Agarose gels were used to visualize migration patterns of DNA samples as per

A


amp^R
ori
bleo^R
KpnI
HindIII
pBAT
10.5 kbp
EcoRV
T
aod-1 3.3 kbp
upstream
BglII
EcoRI

B


+1
+43
....CATCACAAACCTCAAGCGAGTTCCATTACAACTTCACATCACTAGAATTCCGGCCATAGAGCAGCAACC**ATG**....
Remainder of *Spe*I site
*Eco*RI site
Alternative oxidase promoter sequence
Tyrosinase sequence

Sambrook and Russell (2001). For isolation of small DNA fragments (less than 300 bp) for cloning, low melt agarose was used. To pour the gel, 50 mL of normal 1% agarose was melted and poured into the base of the gel casting tray (14.5 cm x 20 cm) and allowed to solidify. The comb was adjusted to place the base of the wells directly on this base. Then 200 mL of 1.2% SeaPlaque agarose (Mandel Scientific Company Ltd, Guelph, ON) was melted, ethidium bromide was added to a final concentration of 0.2 μg/mL, and the solution was poured into the gel casting tray.

2.6 Isolation of DNA from agarose gels

Bands of interest were visualized using a hand held trans-illuminator and excised from agarose gels using a scalpel. DNA was isolated using the glassmilk method (Geneclean II kit, Q Biogene, Carlsbad, CA). When DNA was isolated from low melt gels, the desired bands were purified as per Sambrook and Russell (2001) with modifications. The bands of interest were cut out with a scalpel, melted at 65°C and diluted in TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0) to reduce the concentration of low melt agarose to less than 0.4%. After cooling to room temperature, the mixture was extracted with an equal volume of equilibrated phenol and the aqueous phase was collected. If a large white interface was seen, the interface and phenol layer were re-extracted with an equal volume of TE and this aqueous phase was combined with the first. The total aqueous phase was placed in a fresh 1.6 mL tube and DNA was precipitated by adding one tenth volume 3 M sodium acetate and two volumes of 95% ethanol. This mixture was incubated at room temperature for 10 min and then spun at 14, 000 rpm in a microcentrifuge for 5 min at room temperature. The pellet was washed once with 70% ethanol and then resuspended in an appropriate volume of sterile dH_2O.

2.7 Growth of *E. coli*

Cells of *E. coli* were grown in Luria-Bertani (LB) media (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1 g/L glucose) at 37°C as per Sambrook and Russell (2001). Liquid cultures were shaken at 150-200 rpm to provide aeration of the culture. Filter sterilized antibiotic solutions were added after autoclaving, and 100 mg/L ampicillin (amp) or 70 mg/L kanamycin (kan) were used for selection.

2.8 Transformation of competent *E. coli* cells

Strain XL1, XL2 or XL10 Blue (Stratagene, LaJolla, CA) cells were prepared for transformation as per Inoue (1990) with growth at 18°C to increase competence (Sambrook and Russell, 2001). Aliquots of 250 μL were flash frozen in liquid nitrogen and stored at –80°C until needed.

Competent *E. coli* cells were transformed as per Sambrook and Russell (2001). Cells were thawed on ice and DNA, in a volume of less than one tenth that of the cells, was added. The DNA was mixed with the cells gently, and incubated on ice for 30 min. The mixture was heat shocked either at 42°C for 1.5 min or 37°C for 5 min. One to two volumes of pre-warmed LB were added and the mixture was incubated at 37°C for 30 to 90 min. Aliquots of the transformation mixture were spread onto pre-warmed LB plates containing the appropriate antibiotic. The plates were incubated at 37°C overnight and transformants picked the following day.

2.9 Small scale plasmid or cosmid DNA isolation

E. coli cells containing the DNA construct of interest were grown in 5 or 10 mL LB liquid culture containing the appropriate antibiotic overnight at 37°C. Multiple aliquots (usually two) of 1.5 mL of the culture were poured into a microcentrifuge tube and the cells were pelleted in a microcentrifuge at 14, 000 rpm for 2 min at room temperature. Either the alkaline lysis "miniprep" protocol of Birnboim and Doly (1979) or Qiagen mini-prep kits (Qiagen, Mississauga, ON) were used to isolate the DNA. The Qiagen protocol was followed, including modifications to optimize isolation of low copy number, large insert cosmids, when necessary.

2.10 Large scale plasmid or cosmid isolation

E. coli cells containing the DNA construct of interest were grown in 50 to 500 mL LB liquid culture containing the appropriate antibiotic overnight at 37°C. DNA was isolated using either Qiagen midi- or maxi-prep kits (Qiagen, Mississauga, ON) following the manufacturer's instructions, or by a standard cesium chloride continuous gradient purification as per Sambrook and Russell (2001).

2.11 Determination of nucleic acid concentration

The concentration of DNA or RNA solutions was determined using a Shimadzu UV-Visible Recording Spectrophotometer, Model UV-265 (Shimadzu, Guelph, ON). An appropriate dilution was placed in a quartz cuvette. An OD_{260} of 1.0 for double stranded DNA is equivalent to a concentration of 50 μg/mL, while an OD_{260} of 1.0 for RNA samples is equivalent to a concentration of 40 μg/mL.

2.12 DNA sequencing and analysis

During the early stages of this work, DNA was sequenced using an Amersham-Pharmacia thermosequenase kit (Amersham Pharmacia Biotech, San Francisco, CA) based on a $[^{33}P]$-ddNTP PCR sequencing method. The manufacturer's instructions were followed. In the later stages, DNA sequence was obtained using a DyeNamic sequencing kit system (Amersham Pharmacia Biotech, San Francisco, CA) with a Model 373 stretch sequencer separation system (Applied Biosystems, Foster City, CA). The sequencing reactions and PCR were performed according to the manufacturer's instructions. Sequence profiles were produced using Applied Biosystems sequencing analysis software version 3.4.1 (Applied Biosystems, Foster City, CA). The electrophoresis and determination of sequence profiles were done by the Molecular Biology Service Unit (MBSU; Department of Biological Sciences, University of Alberta).

DNAMAN version 4.13 (Lynnon Biosoft, Vaudreuil, QB) software was used to analyze sequences obtained. Sequences were also analyzed using basic local alignment search tool (BLAST) searches against various databases.

2.13 Oligonucleotides used during this study

Oligonucleotide primers were designed for sequencing and PCR reactions following the guidelines of Sambrook and Russell (2001). Oligonucleotides were synthesized at MBSU (Department of Biological Sciences, University of Alberta) or by Gibco BRL (Burlington, ON) and are listed in Table 5.

Table 5.

Primers used during this study.

Primer	Sequence (5' to 3')	Use of Primer	5' end[1]	3' end
ADE1[*]	TCACTTGCTGGATGGACGC	*T* PCR and Sequencing	-84	-66
ADE20[*]	GGCCATAGAGCAGCAACCATG	*T* Sequencing	+203	+223
ADE19[*]	GGAGGTAGAGATTGAACTGCTCCGG	*T* PCR (Ectopic Reporter)	+402	+378
ADE59[*]	CGTACTTTGATTGGGCTAGTCAGCC	*T* Sequencing	+715	+739
ADE60[*]	CCATGTGTATGTGGTCACTCCCC	*T* Sequencing	+1190	+1212
ADE61[*]	TCTCGGCTGCTGCTGCTCATGCAG	*T* Sequencing	+1678	+1701
ADE62[*]	CCTCGCTTTGCTGCAGGATATTGTG	*T* Sequencing	+2152	+2176
ADE2[*]	TACCCGGTTTCTTGATCTCAC	*T* PCR	+2502	+2482
ADE63[*]	GAAGCTCAGAACTGGATACGACGC	*T* Sequencing	+2632	+2655
ADE58[*]	CTCGAGCCCAACACCAAGAACGTC	*T* PCR	+2908	+2885
ao5	TTAGTTGGGCCGCTTGTCC	*aod-1* PCR and Sequencing	+93	+111
ao21	AGTGATGTGAAGTTGTAATGGA	*aod-1* PCR	+43	+22
FNA88	CCTTCCCTCCAGAAGGCTTTATGCG	*aod-1* PCR and Sequencing	-1216	-1191

[*] Indicates oligonucleotides created during this study.

[1] The 5' and 3' end sites of each primer refers to their positions within the genes for which the primer was designed.

2.14 Polymerase chain reaction (PCR)

PCR was performed as per Sambrook and Russell (2001) using a mixture of *Taq* and *Vent* polymerase (New England Biolabs, Beverly, MA) to minimize replication errors. PCR primers were often designed with the aid of DNAMAN version 4.13 (Lynnon Biosoft, Vaudreuil, QB) software to prevent formation of primer dimers, primers with intramolecular complementarity, and to determine the optimum annealing temperature.

2.15 Conidial DNA preparation for PCR

To obtain small samples of DNA from *N. crassa* strains for PCR, a small, "pea sized," amount of conidia was mixed with 100 μL of cracking buffer (1 M sorbitol, 20 mM EDTA, 3 mg/mL lysing enzyme (Sigma, Oakville, ON). The mixture was incubated at 37°C for 10 min and then spun in a microcentrifuge at 14, 000 rpm for 10 min at room temperature. The pellet was washed with 500 μL 1 M sorbitol, 20 mM EDTA and then resuspended in 100 μL sterile dH_2O. This mixture was then subject to a standard glassmilk DNA isolation procedure (Geneclean II kit, Q Biogene, Carlsbad, CA) and eluted with 50 μL of sterile dH_2O. For a standard PCR reaction, 10 μL of this preparation was used.

2.16 Transformation of *N. crassa* using sphaeroplasts

Sphaeroplasts were prepared as per Akins and Lambowitz (1985) and Schweizer *et al.* (1981). One week old conidia from the strain to be transformed were harvested in sterile dH_2O, filtered through sterile cheesecloth, and the conidia were counted. Liquid cultures were inoculated at 5×10^6 to 1.5×10^7 conidia/mL and grown at 30°C with shaking until at least 80% of the conidia had germinated, typically for 3-6 hr. Germination was monitored by removing small aliquots at various intervals and examining the conidia under a light microscope to visualize small protuberances from the conidia. Cultures were harvested in sterile GS-3 rotor bottles (Sorvall, Mandel Scientific, Guelph, ON) and centrifuged for 10 min at 5, 000 rpm at 4°C. The conidia were kept on ice at all times. The supernatant was removed and the conidia were washed once with cold sterile dH_2O and once with cold sterile 1 M sorbitol (Sigma, Oakville, ON). The conidia were resuspended in cold sterile 1 M sorbitol to a concentration of 1 x 10^9 conidia/mL and transferred to a sterile 50 mL polypropylene centrifuge tube. For each mL of suspended conidia, 4 mg of lysing enzyme (Sigma, Oakville, ON) was added. The mixture was incubated at 30°C with shaking for 15 to 60 min. At 15 min intervals or less, two 10 μL aliquots were removed and placed on a slide to monitor the extent of sphaeroplasting. To one of the aliquots, 10 μL of 10% sodium dodecyl sulfate (SDS) was added. When treated with SDS, conidia whose cell walls have been digested burst and appear under the light microscope as "ghosts" when compared to the untreated sample. The sphaeroplasting was considered complete when at least 90% of the conidia in the treated sample formed ghosts. The suspension was pelleted gently at low speed in a clinical centrifuge (setting 2) for 10 min at 4°C. The supernatant was removed and the sphaeroplasts were washed once with 50 mL sterile cold 1 M sorbitol and once with 50 mL sterile cold MCS (10 mM MOPS, 50 mM $CaCl_2$, 1 M sorbitol, pH 6.3 with NaOH). The sphaeroplasts were resuspended in 6 to 8 mL sterile cold MCS to a final concentration of 2.5 to 5 x 10^8 sphaeroplasts/mL. For each mL of suspended sphaeroplasts, 13 μL of dimethylsulfoxide (DMSO), 65 μL heparin (5 mg/mL), and 275 μL PMC (40% PEG, 10 mM MOPS, pH 6.7 with NaOH, 50 mM $CaCl_2$)[1] were added. The solution was gently and thoroughly mixed, aliquoted, and stored at -80°C.

For transformation, frozen sphaeroplasts were thawed on ice. 5 μg of the DNA to be transformed in a total volume of 60 μL was added to 100 μL of sphaeroplasts. This solution was mixed gently and kept on ice for 30 to 60 min. The mixture was transferred to a sterile 15 mL screw-cap tube and 1.6 mL PMC was added. The solution was gently mixed and incubated at room temperature for at least 20 min. The transformation mixture was added to top agar (1.6% agar, 1 M sorbitol) containing 1 x Vogel's media plus the appropriate supplements and selective agents at approximately 48°C and layered onto appropriate transformation plates. The plates were incubated at 30°C until transformed colonies were visible, typically three to five days. Either resistance to bleomycin or to hygromycin was used as a selectable marker of transformation. For bleomycin selection, both top agar and plate media contained 1 mg/L bleomycin and 0.5 g/L caffeine. Hygromycin selection top agar and plates contained 0.44 mL/L hygromycin.

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[1] The PEG/MOPS and $CaCl_2$ solutions were autoclaved separately and mixed after cooling.

Screening of transformants generated using sphaeroplast transformation with the tyrosinase plate assay (see section 2.27) required that the colonies be on top of, rather than in, the top agar, and that the PMC was completely removed. To accomplish this, after incubation of the transformation mixture at room temperature for 20 min, 50 mL of regeneration media (top agar without the agar) was added and this mixture was incubated at 30°C for 3 hr with gentle shaking. Then the transformed cells were spun down gently at low speed in a clinical centrifuge (setting 2) for 10 min at 4°C. A small amount of sterile 1 M sorbitol was used to resuspend the cells and aliquots were spread on plates made from top agar.

2.17 Transformation of *N. crassa* by electroporation

Transformation by electroporation was performed as per Margolin *et al.* (1997, 2000). One week old conidia of the strain to be transformed were harvested in dH_2O, filtered through sterile cheesecloth, washed three times with cold 1 M sorbitol, and resuspended in cold 1 M sorbitol to a final concentration of $2\text{-}2.5 \times 10^9$ conidia/mL. In a pre-chilled electroporation cuvette, 5 μg of 1 μg/μL linearized transforming DNA was mixed with 40 μL of conidia and left on ice for 5 min. Optimal electroporation settings of 2.1 kV, 475 Ω, 25 microfarads were determined for strain T1P11 using the Gene Pulser (Bio-Rad, Hercules, CA) electroporator (Ian Cleary, personal communication). Electroporation time constants of 11 to 12 msec were obtained. 1 mL of cold 1 M sorbitol was immediately added and the conidia were allowed to recover at 30°C for 30 to 90 min. Various aliquots from 5 to 250 μL of the electroporation mixture were mixed with top agar with the appropriate supplements and selective agents at approximately 48°C and plated on viability plates. Transformants were grown until colonies appeared, usually three to five days at 30°C.

For screening of transformants with the tyrosinase plate assay (see section 2.27), after addition of the 1 M sorbitol, the electroporated conidia were placed in 50 mL regeneration media and incubated at 30°C with gentle shaking for 3 hr. The cells were then pelleted by centrifugation at high speed in a clinical centrifuge (setting 7) for 10 min at 4°C. A small volume of 1 M sorbitol was used to resuspend the cells and aliquots were spread on top agar plates.

2.18 Purification of transformant strains

Transformants were picked by surrounding the colony with the small end of a sterile Pasteur pipette. The colonies were placed in slants containing either full bleomycin or half of the normal concentration of hygromycin (to promote conidiation) and grown at 30°C for two to three days until mycelia covered the surface of the media. The slants were placed at room temperature in the light to promote conidiation, which normally took 4 days to a week. Conidia were then streaked on full concentration bleomycin plus caffeine or hygromycin containing plates, and the plates were incubated at 30°C for two to three days until colonies appeared. Single colonies were picked from these plates as above and placed into slants containing no selective agent. These slants were incubated for two days at 30°C and then placed at room temperature in the light to promote conidiation. This round of purification of single colonies increased the likelihood of isolating homokaryotic transformants.

2.19 *N. crassa* genomic DNA Isolation

gDNA was isolated from 250 mL cultures as described by Wendland *et al.* (1996) with modifications. The overnight culture was harvested by vacuum filtration through a Whatman filter in a Buchner funnel. The remaining mycelial pad was washed with dH_2O and then pressed between paper towels to remove excess moisture. The pad was then immediately wrapped in foil and placed on ice until all cultures were harvested. Each mycelial pad was then weighed. For each gram of mycelia, 1.5 grams of acid washed sand and 2 mL of new isolation buffer (10 mM EDTA pH 8.0, 1% SDS, 100 mM Tris·HCl pH 8.0) were added. This mixture was ground with a mortar and pestle until a smooth consistency was obtained. The slurry was placed in a Sorvall SS-34 tube and brought up to 10 mL total volume with new isolation buffer. The tube was shaken vigorously and then mixed by inversion for at least 1 minute. The slurry was incubated at 70°C for one hour and then chilled on ice for 10 min. After chilling, 0.64 mL of 8 M potassium acetate pH 4.3 was added, the sample was mixed by inversion, and the slurry kept on ice for 1 hour. The sample was spun at 14, 000 rpm for 15 min at 4°C in an SS-34 rotor (Sorvall, Mandel Scientific, Guelph, ON) and the supernatant was transferred to a new tube. An equal volume of isopropanol was added to the supernatant and the tube was mixed gently. A visible clot of DNA and RNA then formed. The sample was spun at 10, 000 rpm for 10 min at 4°C in an SS-34 rotor (Sorvall, Mandel Scientific, Guelph, ON), and 1 mL of 70% ethanol was added and poured off to wash the pellet. The pellet was dissolved in 400 μL 1 mM EDTA pH 8.0. The solution was placed in a microcentrifuge tube and 200 μL of high salt buffer (100 mM sodium chloride, 25 mM Tris·HCl pH 7.4, 2 mM EDTA) and 15 μL boiled RNase A (10 mg/mL) were added. After mixing, the sample was incubated at 37°C for 30 min. The mixture was extracted with an equal volume of equilibrated phenol. DNA was precipitated by adding one tenth volume 3 M sodium acetate and two volumes of 95% ethanol. A visible clot formed and was removed from the 1.6 mL tube using a 1 mL disposable pipette tip. The clot was washed by briefly placing it in a 1.6 mL tube with 70% ethanol. Then the clot was placed in a new microcentrifuge tube and allowed to dry. The DNA was resuspended in 400 μL sterile dH_2O and stored at –20°C.

2.20 Genomic DNA gels and Southern blotting

Samples of gDNA were digested, subjected to electrophoresis on agarose gels, and transferred to nylon membranes as per Sambrook and Rusell (2001) with modifications. 5 μg of each genomic DNA sample was digested with the appropriate enzyme (or enzymes) overnight in an air incubator at the suitable temperature in a total volume of at least 40 μL. Loading dye was added and the samples were run in a 0.6% agarose, 0.2 μg/mL ethidium bromide gel to resolve larger fragments. A lambda *Hin*dIII marker lane was also subjected to electrophoresis as a size standard. The gel was run at 150 V for 3 hr, and the running buffer was replaced at 1.5 hr. Following electrophoresis, a picture was taken of the gel using a transilluminator and a fluorescent ruler. The gel was incubated with 0.25 M hydrochloric acid for 10 to 15 min with slow shaking until the loading dye had changed from blue to yellow. The gel was then washed with dH_2O, and the DNA was denatured by placing the gel in 1.5 M sodium chloride, 0.5 M sodium hydroxide twice for 20 min each with slow shaking. The gel was washed with dH_2O and placed in a neutralizing 3 M sodium acetate solution for 30 min with gentle shaking. The gel was rinsed in dH_2O and placed, wells facing down, on Saran wrap. A nylon membrane the same size as the gel was wetted and then placed on the gel. Any bubbles were removed by rolling a 25 mL glass pipette along the nylon. A piece of 3MM paper the same size as the membrane, wetted with 20 x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), was placed on top of the membrane followed by 2 to 5 more sheets of un-wetted 3MM paper of the same size. A stack of paper towels of the same size was placed on top of the 3MM paper, followed by a glass plate and then a flask containing 500 mL water. The DNA transfer was left overnight. The next morning, the wells of the gel were marked on the nylon membrane, which was then washed for 5 min in 2 x SSC. The membrane was blotted dry on 3MM paper and baked for 1 hour at 80°C under vacuum.

2.21 Southern hybridization

Nylon membranes were pre-hybridized for 6 hr to overnight in pre-hybridization solution (10 x Denhardts, 2 x SSC, 1% SDS, 0.1 mg/mL sonicated denatured salmon sperm DNA) at 65°C in a rotating hybridization incubator (Tyler Research Instruments, Edmonton, AB). Purified, radioactively labeled probe (see sections 2.22, 2.23) was denatured by incubation at 100°C for 10 min and then placed in 20 mL of fresh pre-hybridization solution and added to the membrane. Hybridization was done at 65°C overnight in the rotating oven. The membrane was then washed twice for 5 min at room temperature with 100 mL of 2 x SSC, 0.1% SDS, and twice for 20 min at 65°C with pre-warmed 2 x SSC, 2% SDS. Finally, the membrane was washed twice for 20 min at 65°C with pre-warmed 0.1 x SSC, 0.1% SDS. The membrane was wrapped in Saran wrap and exposed to Kodak XAR film at –80°C for an appropriate amount of time.

2.22 Radioactive labeling of DNA

Probe DNA was denatured for 10 min at 100°C and then cooled in an ice/salt bath. The DNA was diluted to a volume of 50 μL and mixed with 12 μL of a dNTP mix (0.5 mM dATP, 0.5 mM dGTP and 0.5 mM dTTP), 8 μL of a hexanucleotide mix (Roche Scientific, Laval, QB), 5 μL of ^{32}P dCTP, and 4 μL of Klenow DNA polymerase. The labeling reaction was incubated at 37°C for 30 to 60 min. Unincorporated nucleotides were removed using a Sephadex G50 spin column (see section 2.23).

2.23 Sephadex G50 columns for probe purification

Sephadex columns were used to remove unincorporated radioactive nucleotides as per Sambrook and Russell (2001). Sephadex G50 was mixed with sterile TE and left at room temperature overnight. The Sephadex was swollen by autoclaving at 121°C, 21 psi for 20 min. To make a spin column, a 1 mL syringe was plugged with silanized glass wool and the Sephadex slurry was added. The syringe was placed in a 15 mL screw-cap tube and spun at top speed for 2 min in a clinical centrifuge. A second addition of slurry and a second spin were done to increase the column length. The TE flow-through from the column was removed before the labeling reaction was placed on the surface of the Sephadex bed. A 1.6 mL tube was placed at the bottom of the screw-cap tube to collect the labeled DNA probe in the flow-through. The column was centrifuged at top speed for 2 min in the clinical centrifuge, and the flow-through in the 1.6 mL tube was collected.

2.24 RNA isolation

All equipment used for RNA isolation and analysis was either soaked overnight in 2% hydrogen peroxide or washed with RBS35 detergent (Chemical Products R. Borghgraef, Pierce Rockford, IL) and then rinsed with milliQ dH_2O. Reagents were made using diethylpyrocarbonate (DEPC) treated dH_2O, where dH_2O with 0.1% DEPC was stirred at room temperature overnight and then autoclaved at 121°C, 21 PSI for 20 min. Prior to RNA isolation, a respiration assay of the culture of interest was performed (see section 2.29) followed by harvesting of the mycelia. A portion of the mycelial pad was immediately placed in foil and frozen in liquid nitrogen. These pads were stored at –80°C until used for RNA isolation. Roughly 100 mg pieces of the pad were ground in liquid nitrogen using a mortar and pestle. RNA was isolated using a Qiagen RNeasy Plant Mini Kit (Qiagen, Mississauga, ON) following the kit directions. RNA was eluted in two volumes of 50 µL RNase-free water.

2.25 RNA gels and Northern blotting

1% Agarose gels containing 1.9% formaldehyde were used with a 1 x MOPS-EDTA (0.02 M MOPS, 2 mM sodium acetate, 1 mM EDTA) running buffer. 5 µg of each RNA sample was placed in loading buffer (1 x MOPS-EDTA, 50% formamide, 6.5%

formaldehyde, 0.1 mg/mL ethidium bromide) and heated to 65°C before loading. The gels were run at 150 V for three hr in a fume hood, with periodic manual stirring of the running buffer. The gels were photographed, washed three times for 20 min in DEPC treated dH_2O, soaked in 0.05 N sodium hydroxide for 20 min, and finally neutralized in 10 x SSC for 40 min. A nylon membrane of the same size as the gel was wetted in 10 x SSC for five min. The RNA from the gel was transferred to the nylon membrane overnight by capillary action using 10 x SSC as the transfer buffer. After transfer, the wells from the gel were marked on the nylon membrane, which was then rinsed twice in 2 x SSC for five min and baked at 80°C in a vacuum oven for 1 hour.

2.26 Northern hybridization

The membrane was pre-hybridized with 50 mL Church's Buffer (0.5 M sodium phosphate pH 7.5, 1 mM EDTA, 7% SDS) at 65°C in a rotating incubator. The membrane was probed with a labeled 1.3 kbp *Eco*RI/*Spe*I cDNA fragment of *aod-1* corresponding to –50 to +1465 of *aod-1*. The probe was denatured for 10 min at 100°C and then added to 25 mL fresh Church's Buffer. The blot was hybridized in this solution overnight at 65°C, then washed with 1 x SSC, 0.1% SDS four times for 5 min at room temperature, and finally with pre-warmed 0.1 x SSC, 0.1% SDS twice for 25 min at 65°C. The Saran wrapped membrane was exposed to Kodak XAR film at –80°C.

2.27 Tyrosinase plate assay

Conidia from strains to be tested were streaked on plates to obtain 50 to 100 colonies per plate. For assays of non-induced colonies, the plates contained minimal medium with appropriate supplements and were grown at 30°C for two days. To induce reporter expression, the plates contained chloramphenicol and required an additional day of growth at 30°C. Strain NCN10 was used to optimize the assay conditions using only the relatively low level of endogenous tyrosinase. It was found that maximal formation of the brown pigment occurred when 5 to 10 mL of a freshly prepared 10 mM tyrosine 0.1% Triton X-100 solution was placed on the plates, and the plates were maintained at 30°C for several hr to overnight. Triton X-100 is presumed to facilitate the proteolytic cleavage required for tyrosinase maturation by making the cellular compartments leaky, which exposes the protyrosinase to protease(s) (S. Free, personal communication). By plating and testing mixtures of NCN10 and T1P11 condia, it was found that brown and white colonies could be distinguished, although after 1-2 days the brown pigment usually leaked into the media, turning it and any white colonies brown.

2.28 Insertional mutagenesis

Transformation of *N. crassa* usually results in the integration of the foreign DNA at ectopic sites within the genome (Davis, 2000). A mutagenesis scheme was designed based on these random integration events, since insertion of foreign sequences can interrupt regulatory and/or coding sequences and cause loss of function mutations. Plasmid pCSN43, which carries a hygromycin resistance marker, was used for insertional mutagenesis of strain 8-18. The strain was transformed (see sections 2.16 and 2.17) with *Hin*dIII cut pCSN43, and plated on top of hygromycin-containing plates or hygromycin and chloramphenicol -containing plates. After four or five days growth at 30°C, colonies were subjected to the tyrosinase plate assay.

2.29 Ethyl methanesulfonate (EMS) mutagenesis and enrichment

Conidia from the reporter strain T11-76 were treated with EMS using a method adapted from Davis and de Serres (1970; Fig. 6). Seven to ten day old conidia flasks were harvested in sterile dH_2O and filtered through cheesecloth to remove mycelia. The conidia were then washed once in fresh sterile dH_2O and resuspended in 0.067 M phosphate buffer pH 7.0 at a concentration of 2×10^7 conidia/mL. Tests of the effectiveness of EMS were carried out using final concentrations of 0.1, 0.15, or 0.3 M. I found that concentrations of EMS higher than the recommended 0.1 M were necessary to reduce survival to the level of 70% suggested to be optimal for mutagenesis (Table 6; Davis and de Serres, 1970). Although desired mutations were obtained using all three EMS concentrations, it was found that 0.15 and 0.3 M EMS were more effective at generating mutations than when 0.1 M EMS was used. In the results section (section 3.8), the experiments using all three concentrations of EMS are grouped together when discussing the number and identity of mutations obtained.

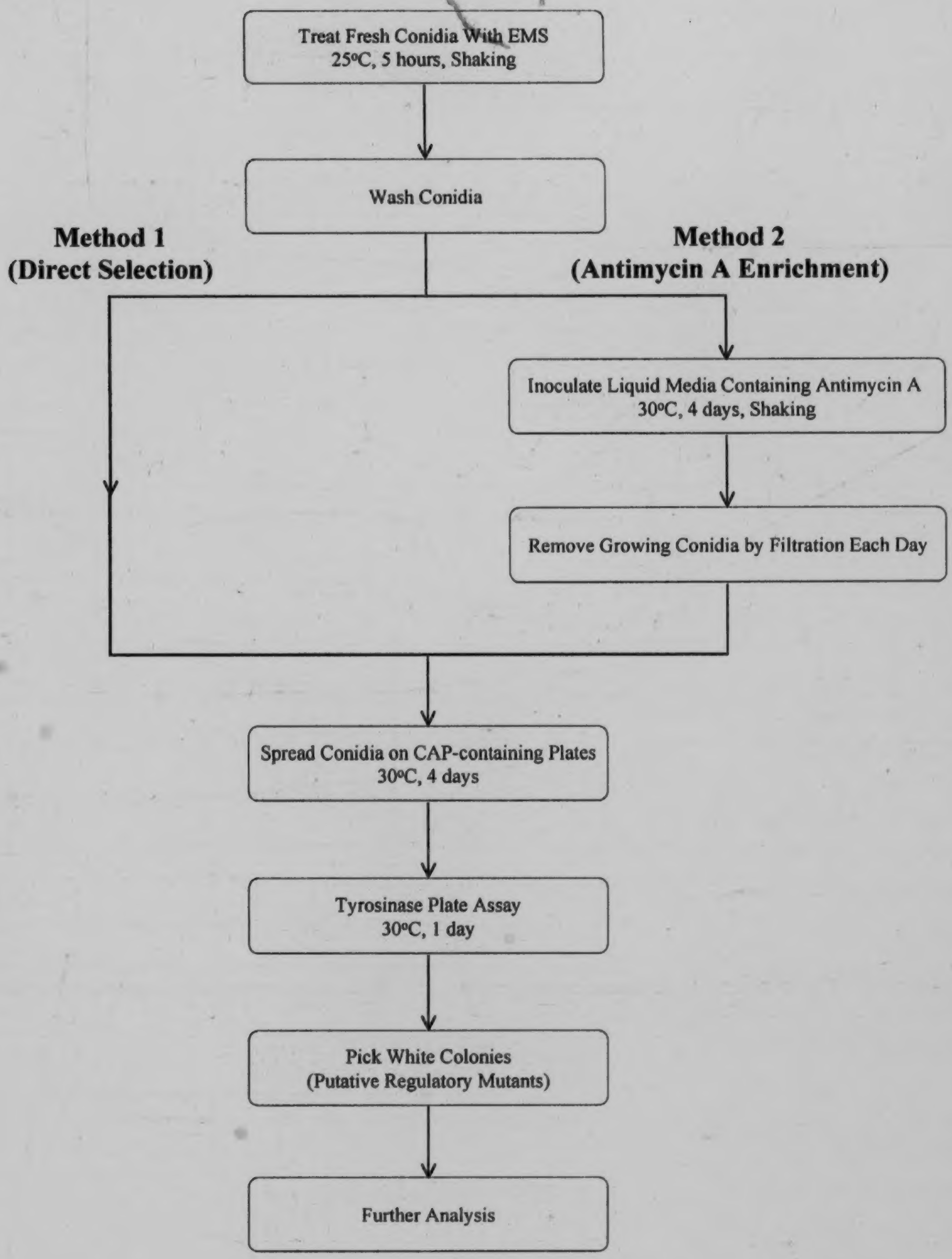

Treat Fresh Conidia With EMS
25ºC, 5 hours, Shaking
Wash Conidia
Method 1
(Direct Selection)
Method 2
(Antimycin A Enrichment)
Inoculate Liquid Media Containing Antimycin A
30ºC, 4 days, Shaking
Remove Growing Conidia by Filtration Each Day
Spread Conidia on CAP-containing Plates
30ºC, 4 days
Tyrosinase Plate Assay
30ºC, 1 day
Pick White Colonies
(Putative Regulatory Mutants)
Further Analysis

Table 6.

Tests of conidial survival after treatment with different concentrations of EMS.

EMS Concentration	Number of Conidia Plated	Number of Colonies (No EMS)	Number of Colonies (After EMS Treatment)	% Survival
0.1 M	500	300	282	94
	500	295	244	83
	500	313	303	97
0.15 M	200	125	77	62
	200	87	72	83
	200	139	106	76
	100	64	45	70
	100	43	31	72
0.3 M	300	173	59	34
	200	139	26	19
	100	57	13	23

Conidia were incubated with EMS at 25°C for five hr with shaking and then washed twice with sterile 1 x Vogel's solution. Two methods were used for the selection of mutants (Fig. 6). For the direct selection method, EMS treated conidia were spread directly onto chloramphenicol containing plates. Varying amounts of conidia were diluted in 1 x Vogel's and spread to obtain between 50 and 100 viable colonies per plate. Plates were incubated at 30°C for four days, and then colonies were screened by the tyrosinase assay. For the antimycin A enrichment method, EMS treated conidia were first placed in antimycin A liquid media (0.5 μg/mL) for four days growth and then plated as above. 2 x 10^7 EMS treated conidia were placed in 500 mL of media containing antimycin A (0. 5 μg/L) in a 2 L baffled flask and incubated at 30°C with shaking for four days. Every 24 hr the liquid cultures were filtered through sterile nylon stocking to remove any growing conidia with hyphal extensions. Thus, these cultures were filter enriched for antimycin A sensitive mutants. After four days, the remaining conidia were collected and plated on chloramphenicol containing plates. Again, plates were incubated at 30°C for four days and the resulting colonies were screened using the tyrosinase plate assay. In both cases, colonies that remained white during the tyrosinase plate assay were picked.

2.30 Respiration assay

Conidia of strains to be tested were harvested and used to inoculate liquid cultures for overnight growth at 30°C with shaking. Depending on the concentration of the mycelia grown (estimated by visual inspection), 1 to 3 mL of fresh mycelia was removed from the culture and brought up to 3 mL total volume with 1 x Vogel's in a glass chamber. Air was bubbled through this mixture for at least 1 minute. Consumption of oxygen was measured using a Yellow Springs Instruments biological oxygen monitor (Model 53, YSI, Yellow Springs, OH) with a Clark type oxygen sensor. 50 μL of freshly prepared potassium cyanide (0.2 M in 10 mM Tris·HCl pH 7.2, 5 mM EDTA) or SHAM (0.3 M in 95% ethanol) was added after an initial respiration rate was established to observe their affects on oxygen consumption.

2.31 Mating strains and isolating progeny

Crossing was performed as described by Davis and de Serres (1970). Briefly, the strain to become the female parent was inoculated on Westergaard's media (low nitrogen) using filter paper as the sole carbon source. When necessary, the media contained normal concentrations of nutritional supplements. However, if amino acids were required, only 1/50 the normal amount was added to cultures to prevent an increase in nitrogen concentration that would inhibit protoperithecial formation. Crossing plates were incubated at 30°C for two days and then kept at room temperature in the dark until protoperithecia formed. The male strain was inoculated on slants and allowed to conidiate. Conidia were harvested and spread on the protoperithecia to allow mating to occur. After application of the male strain conidia, the plates were kept at room temperature in the light. Four days after mating, the plate lids were removed and clean lids were put in place. Ascospores were harvested from the lids eighteen days after mating by suspension in sterile dH_20, left to hydrate in sterile dH_2O overnight at room temperature, and then activated at 60°C for 45 to 60 min. Aliquots of 5 to 250 μL of the ascospore suspension were plated on sorbose containing media and incubated at 30°C overnight. Using a dissecting microscope, individual ascospores showing hyphal growth were picked (see section 2.18). Care was taken to avoid picking ascospores whose hyphae overlapped with hyphae of another ascospore.

2.32 Growth rate measurement

Race tubes were made in sterile 25 mL plastic pipettes (White and Woodward, 1995) by filling each tube with 13 mL of 1 x Vogel's media (with 1.6% agar and appropriate supplements) and laying the tubes flat to allow the media to solidify. Fresh conidia were harvested from each strain and 1×10^5 conidia were inoculated at one end of each tube. Strains were grown in triplicate at either 30°C or room temperature (about 22°C), and the extent of mycelial growth was measured daily. Growth rate was graphed for three days at 30°C or four days at room temperature. Standard error was calculated for each growth point.

2.33 Mitochondrial isolation

Mitochondria were obtained as described (Pfanner and Neupert, 1985). Fresh cultures were harvested by filtration through a Whatman filter in a Buchner funnel. Mycelial pads were washed with dH_2O and pressed between paper towels to remove excess liquid. If desired, a small portion of the mycelial pad was immediately frozen in liquid nitrogen for RNA isolation. The remaining pads were kept in foil on ice until further processed. For each gram of mycelia, 1 mL of SEMP buffer (0.25 M sucrose, 1 mM $EDTA \cdot Na_2 \cdot 2H_2O$, 10 mM MOPS, pH 7.2 (with potassium hydroxide), with freshly added phenylmethylsulfonyl fluoride to 1 mM) and 1 g acid washed sand were added. Pads were ground on ice using a mortar and pestle until an even consistency was reached. A second equivalent volume of SEMP solution was added and the sample ground again. The slurry was placed in a Sorvall SS-34 tube and spun at 5, 000 rpm for 5 min at 4°C. The supernatant was poured into a new tube, which was then spun at 12, 000 rpm for 20 min at 4°C. This supernatant was discarded and the mitochondrial pellet was resuspended in a small volume of SEMP buffer. Protein concentrations were determined using the Bradford method with BioRad reagents (Mississauga, ON) as per manufacturer's instructions. Mitochondrial preparations were used immediately or stored at – 80°C.

2.34 Mitochondrial purification

Mitochondria to be used for 2-dimensional (2-D) gel electrophoresis (see section 2.37) were isolated as described in section 2.32 and then purified on a three step flotation gradient consisting of 60%, 55% and 45% sucrose in SEMP buffer. The crude mitochondrial pellet was resuspended in a few drops of 60% SEMP, and additional 60% SEMP was added in small aliquots with mixing each time until 10 mL of 60% SEMP in total were added to the mitochondrial suspension. A drop of this mixture was placed on top of 1 mL 55% SEMP and watched for movement to the bottom of the liquid to insure that the density of the mitochondrial solution was greater than the 55% SEMP solution. The 60% sucrose mitochondrial suspension was then placed in a Beckman SW28 swinging bucket rotor tube. On top of the mitochondrial suspension, 10 mL of 55% SEMP was carefully layered to prevent mixing of the layers. Finally, 17 mL of 45% SEMP was carefully layered on top. Tubes were spun in a Beckman ultracentrifuge at

25, 000 rpm for 2 hr and 10 min at 4°C. The mitochondrial band was found at the interface of the 55% and 45% layers and was carefully removed with a 1 mL pipettor. The mitochondria were placed in an SS-34 tube and gently mixed with about two volumes of SEMP. The mitochondria were pelleted at 12, 000 rpm for 20 min at 4°C, and the pellet was resuspended in a small volume of SEMP buffer. Protein concentrations were determined using the Bradford method with BioRad reagents (Mississauga, ON) as per manufacturer's instructions. Mitochondrial preparations were frozen at – 80°C.

Prior to running the 2-D gels, the samples were further purified to remove contaminants (such as mitochondrial DNA, RNA and lipids) that had affected resolution on initial trials. A PlusOne 2-D Clean-Up Kit was generously donated by Amersham Pharmacia Biotech (San Francisco, CA) and the manufacturer's directions were followed.

2.35 Cytochrome spectra

Spectra were obtained as described by Bertrand and Pittenger (1969). Mitochondrial preparations in 250 to 500 μL SEMP containing 5 to 13 mg of protein were brought up to a volume of 2 mL with 2.5% deoxycholate (in 10 mM Tris·HCl, 5 mM EDTA). The samples were mixed by inversion and then spun in a microcentrifuge at 14, 000 rpm for 2 min. The supernatant was divided equally into two cuvettes. A base line comparison between the two halves of the sample was taken first. Then a few crystals of potassium ferricyanide were added to fully oxidize the reference cuvette, and a few crystals of sodium hydrosulfite were added to fully reduce the sample cuvette. Both cuvettes were mixed by inversion until the crystals had dissolved. Difference spectra were obtained from 500 to 650 nm using a Shimadzu UV-Visible Recording Spectrophotometer, Model UV-265 (Shimadzu, Guelph, ON).

2.36 SDS-PAGE and Western blotting

Samples of 25 μg of mitochondrial protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gels (29:1 acrylamide: bis-acrylamide) as per Sambrook and Russell (2001). The gels were soaked in transfer buffer (0.025 M Tris base, 0.19 M glycine, 20% methanol) for 30 min, and then electroblotted to pre-wetted nitrocellulose membranes (Osmonics Inc., Westborough, MA) with transfer buffer using a liquid transfer method (Biorad, Mississauga, ON). Transfer was done at 67 V for 90 min.

2.37 Immunological protein detection (Western blot analysis)

Membranes were blocked for 1 hour to overnight in milk buffer (5% skim milk powder in TBS-Tween Buffer: 0.02 M Tris·HCl pH 7.5, 0.15 M sodium chloride, 0.5% Tween). Primary antibodies raised in rabbits were diluted appropriately in milk buffer containing 0.02% sodium azide. The membrane was incubated with the primary antibody for at least one hr with gentle shaking, followed by three 5 min washes with TBS-Tween Buffer. The secondary antibody (goat anti-rabbit antibody HRP-conjugate, Biorad, Mississauga, ON) was diluted 1:3000 in milk buffer. The membrane was incubated with the secondary antibody for 20 to 30 min and then washed twice for 5 min with TBS-Tween Buffer, once with TBS Buffer alone (as above but no Tween), and once in dH_2O. Bound antibody was detected using the LumiGLO chemiluminescent kit (Mandel, Guelph, ON), and exposed to Kodak XAR film.

2.38 Two-Dimensional gel electrophoresis

The mitochondrial proteome of various strains was analyzed by 2-D electrophoresis following the instructions of Amersham Pharmacia Biotech (Berkelman and Stenstedt, 1998; San Francisco, CA). After processing 150 μg of mitochondrial protein with the PlusOne Clean-Up Kit (see section 2.34), samples were dried at 37°C for 5 min and then resuspended in 250 μL of rehydration buffer (7.2 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH range 3-10, trace bromophenol blue) and 9.5 μL 1 M DTT at 37°C for 10 min. The rehydration buffer contained thiourea to help solubilize membrane proteins (Rabilloud, 1998). The solution was left at room temperature for 50 min and then spun in a microcentrifuge at 14, 000 rpm for 10 min at room temperature. The supernatant was transferred to a fresh tube. The samples were placed in the first dimension strip holders by pipetting carefully into the middle of the holder to prevent the formation of bubbles. The precast immobilized pH gradient (IPG) used was a pH 3-10 linear 13 cm strip, and was placed gel side down into the sample starting at the anode end and slowly lowered to prevent bubbles. Sterile mineral oil was placed on top of the gel strip. Rehydration and isoelectric focusing were performed over the course of 24 hr in an IPGphor isoelectric focusing system (Amersham Pharmacia Biotech, San Francisco, CA). The program consisted of 8 hr at 30 V, 8 hr at 60 V, 1 hour at 200 V, 1 hour at 500 V, 1 hour at 1000 V and 5 hr at 8000 V. A total of 35, 386 to 40, 992 Vhr were obtained for the samples analyzed. The gel strips were equilibrated in 10 mL SDS equilibration buffer (50 mM Tris·HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, trace bromophenol blue, with 100 mg DTT freshly added) for 10 min at room temperature with gentle shaking. The strip was then carefully placed at the top of a 12.5% SDS-PAG with no air bubbles between the gels. The strip was sealed in place by adding melted agarose solution (0.5%) at 60°C. The second dimension gels were run at 35 V, 15 mA for 16.5 hr. The anodic side of the first dimension gel appears at the left on all figures.

2.39 Silver staining

Silver staining of 2-D gels was done using a modified protocol (Blum *et al.*, 1987). All incubations included gentle shaking of the gel in the solution. The gel was fixed in a 50% methanol, 10% acetic acid solution (v/v) for 30 min at room temperature. The gel was then placed in 50% methanol for 15 min at room temperature. This was followed by five washes of 5 min each in milliQ purified dH_2O, and the gel was then placed into fresh sodium thiosulfate solution (0.2 g/L) for 1 min at room temperature. The gel was then washed twice in milliQ dH_2O for 1 min at room temperature. Next, a silver nitrate solution (1 g/L) chilled to 4°C was added and the gel was incubated at 4°C for 25 min, followed by two washes in milliQ pure dH_2O for 1 min each at room temperature. A fresh anhydrous sodium carbonate solution (20 g/L) containing 37% formaldehyde was added two to three times for 3-5 min each at room temperature until the desired staining was achieved. An EDTA solution (14 g/L) was added for 10 min at room temperature to stop the development. Finally, the gel was washed twice for 1 min in milliQ dH_2O.

2.40 MALDI-TOF mass spectroscopy sequencing

Protein spots of interest were excised from silver stained gels using a wide-bored pipette tip and placed in sterile dH_2O. The spots were digested with trypsin and the masses of the fragments determined by the Institute for Biomolecular Design

(Department of Biochemistry, University of Alberta) using matrix-assisted laser desorption ionization – time of flight (MALDI-TOF). The masses were then used in an MS/MS ion search using the Mascot search engine (www.matrixscience.com) against various databases.

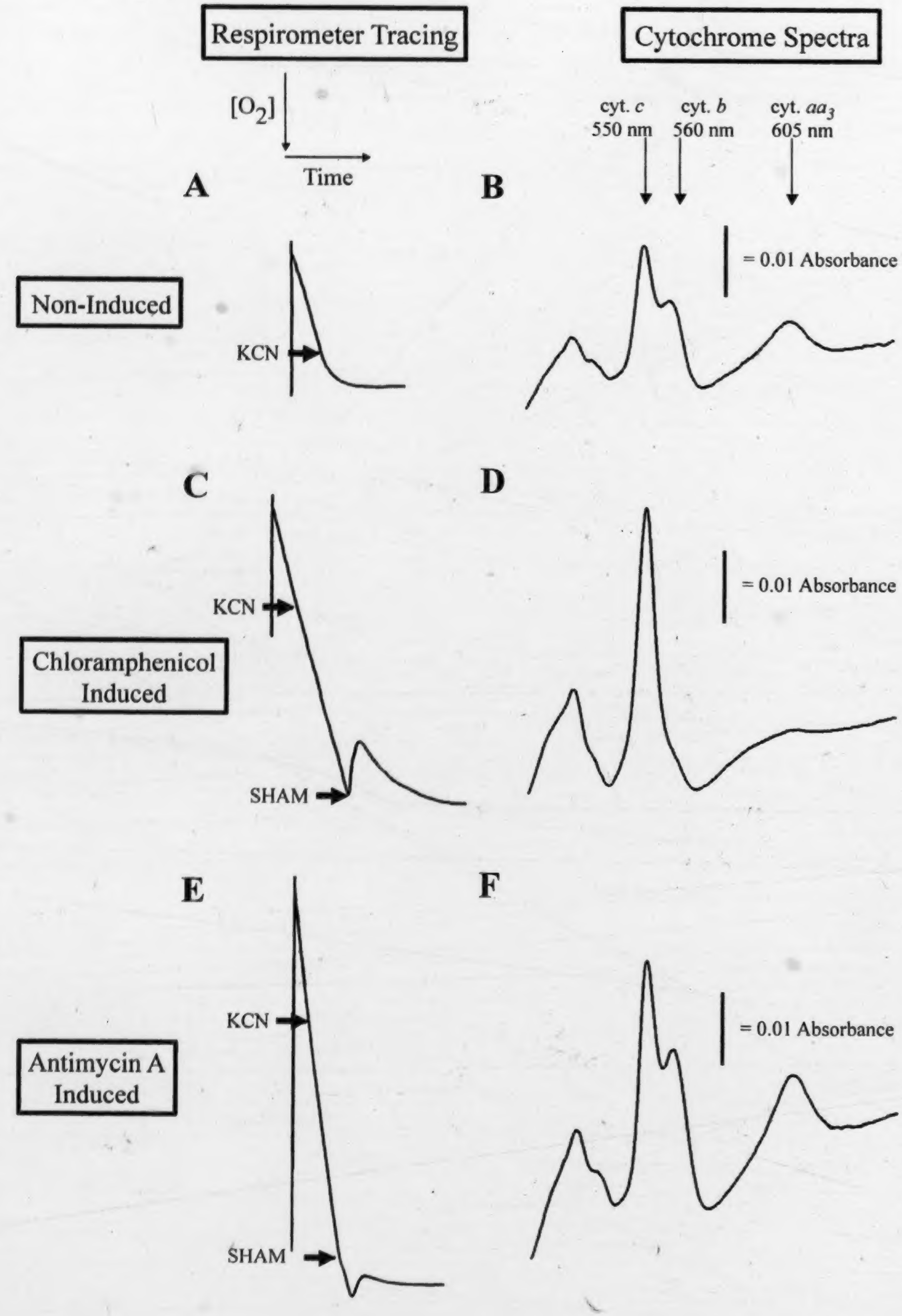
3 Results

3.1 Assay for alternative oxidase activity

Growth of *N. crassa* in the presence of chloramphenicol or antimycin A causes induction of the alternative pathway of respiration in response to the inhibition of the cytochrome pathway (see section 1.6; Fig. 1; section 1.9; Lambowitz and Slayman, 1971). Much of the data in this thesis depends on the detection of alternative oxidase activity following growth of cells under various conditions. Therefore, the respiratory assay and the use and effects of specific respiratory inhibitors are briefly described.

The presence of alternative oxidase activity can be assayed by a respirometer, which records the consumption of oxygen over time (Fig. 7A, C, E). During a respiration assay, the addition of cyanide, an inhibitor of complex IV (see section 1.6), or SHAM, an inhibitor of alternative oxidase (see section 1.7), reveals whether respiration is occurring through the cytochrome pathway or through the alternative oxidase, respectively. As shown in Fig 7A, addition of cyanide to wild-type cells grown under normal conditions (also referred to in this thesis as non-inducing conditions) results in a cessation of respiration, demonstrating that this culture was respiring exclusively through the cytochrome pathway.

Alternative oxidase activity can be induced in wild-type cells by growth in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis (Schlünzen *et al.*, 2001). The respiration of cells grown in the presence of chloramphenicol is not stopped by the addition of cyanide, but is blocked when SHAM is also added to the culture, indicating respiration through the alternative pathway (Fig. 7C). Since chloramphenicol prevents the translation of mtDNA encoded apocytochrome *b* and subunits of complex IV (see section 1.3.1), the amount of cytochromes *b* and aa_3 are reduced when compared to non-induced cultures (compare panels B and D of Fig. 7). The inhibition by chloramphenicol is incomplete, as mutant strains that lack alternative respiration are still able to grow in the presence of chloramphenicol (see section 3.11). Addition of antimycin A to cultures also induces alternative respiration. Antimycin A blocks the flow of electrons through complex III by binding cytochrome b_{560} (see section 1.6). Growth in the presence of antimycin A also results in respiration that is insensitive


Respirometer Tracing
Cytochrome Spectra
[O$_2$]
Time
cyt. *c*
550 nm
cyt. *b*
560 nm
cyt. *aa$_3$*
605 nm
A
B
= 0.01 Absorbance
Non-Induced
KCN
C
D
KCN
Chloramphenicol Induced
SHAM
= 0.01 Absorbance
E
F
KCN
Antimycin A Induced
SHAM
= 0.01 Absorbance

to the addition of cyanide alone, but sensitive to the further addition of SHAM, demonstrating that the culture was respiring through alternative oxidase (Fig. 7E). Unlike chloramphenicol, antimycin A does not result in decreased levels of the cytochrome constituents of the electron transport chain (Fig. 7F).

3.2 Initial attempts to identify factors involved in alternative oxidase expression

At the beginning of my thesis work, I attempted to isolate and identify genes involved in regulating alternative oxidase expression using a yeast one hybrid approach and by assaying for alternative oxidase activity in previously identified signaling mutant strains of *N. crassa*. These projects were unsuccessful but are described briefly as the negative results are somewhat informative.

3.2.1 Yeast one hybrid analysis

Previous work had shown that the CRE found 746 bp upstream of the transcription start site of *aod-1* bound a protein (Tanton *et al.*, 2003). To identify the gene encoding the protein that bound to this sequence, I attempted a yeast one hybrid screen. In this system, the target DNA is placed upstream of a yeast reporter gene and the construct is integrated into the genome of an appropriate yeast strain. An expression library from the organism of interest is then transformed into the reporter strain to identify clones encoding protein(s) able to bind the target sequence and activate expression of the reporter.

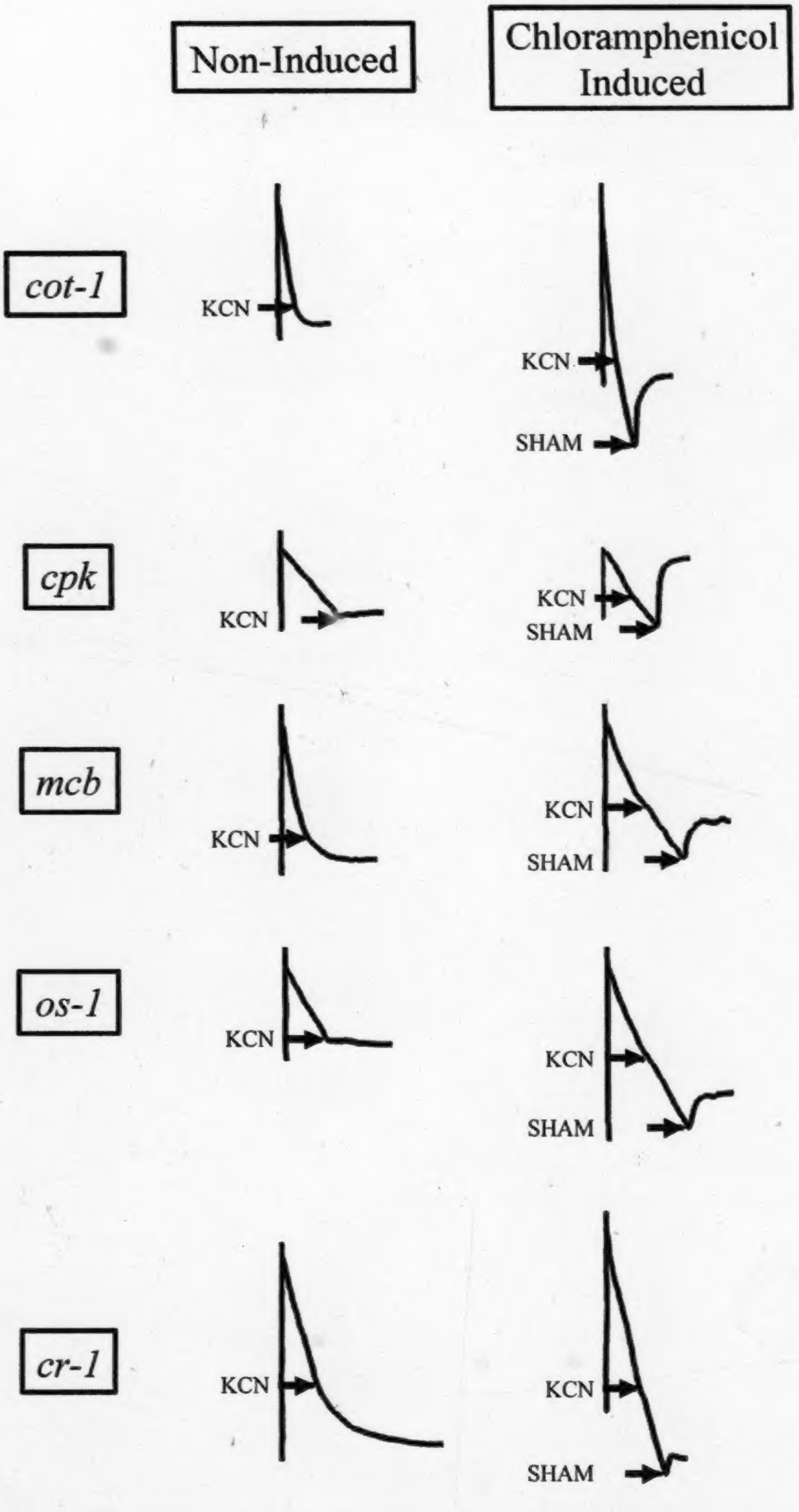
I cloned 4 tandem copies of a 31 bp fragment of *aod-1* upstream sequence that contained the CRE into the one hybrid vector pHisi-1 to create the construct pCHisi-1. In this vector, the *HIS3* gene is used for both positive selection of transformants and as the reporter gene. Leaky expression of *HIS3* allows the selection of transformants carrying the reporter and several yeast transformant strains that had integrated the construct were selected based on growth in the absence of histidine. The next step was to determine the optimal concentration of 3-AT (3-amino-1,2,4-triazole), an inhibitor of His3p, specific for the reporter strain. Sufficient 3-AT must be added to inhibit growth due to leaky reporter expression while still allowing high levels of expression of *HIS3*, which should be reached when the reporter system is activated by an introduced activating construct, to facilitate growth in the absence of histidine. The correct concentration of 3-AT would then be used during screening of the expression library. A control yeast transformant strain carrying the *HIS3* gene under the control of 3 tandem copies of the p53 binding site was made and compared to the experimental reporter strain. While the control strain was unable to grow in 15 mM 3-AT, the pCHisi-1 strain was able to grow in 100 mM 3-AT, the highest concentration tested. It is possible that a yeast protein was able to bind the target sequence and cause high levels of *HIS3* expression. Since the "leaky" reporter background expression was too high to perform the expression library screen, this approach was discontinued.

3.2.2 Alternative oxidase activity in kinase mutants

A signal transduction pathway from mitochondria to the nucleus, responsible for inducing *aod-1* expression, could involve the activity of kinases or signaling components (see section 1.10). Therefore, previously characterized strains of *N. crassa* carrying mutations in genes involved in two-component or cAMP signaling were obtained and analyzed for their ability to properly induce alternative oxidase activity. The mutant strains *cr-1* (an adenylate cyclase mutant), *cot-1* (a cAMP-dependent protein kinase A (PKA) mutant), *cpk* (a strain with PKA activity that is independent of cAMP), *mcb* (a PKA regulatory subunit mutant), and *os-1/nik-1* (a two-component signaling histidine kinase mutant) were assayed for respiration. These strains were all able to normally regulate alternative oxidase activity, showing cyanide-sensitive respiration when grown in non-inducing conditions and cyanide-insensitive respiration when grown in the presence of chloramphenicol (Fig. 8). Thus, alternative oxidase regulation in chloramphenicol induced cultures does not involve the *os-1/nik-1* two component signaling pathway or a cAMP dependent signaling pathway.

3.3 Selection scheme for isolating alternative oxidase regulatory mutants

Previous efforts to identify genes involved in alternative oxidase regulation in *N. crassa* have used the absence of alternative oxidase activity itself as the basis of selection (Edwards *et al.*, 1976; Bertrand *et al.*, 1983). This approach resulted in the isolation of 22 *aod-1* and 2 *aod-2* mutant strains, indicating a clear bias towards selection of mutations

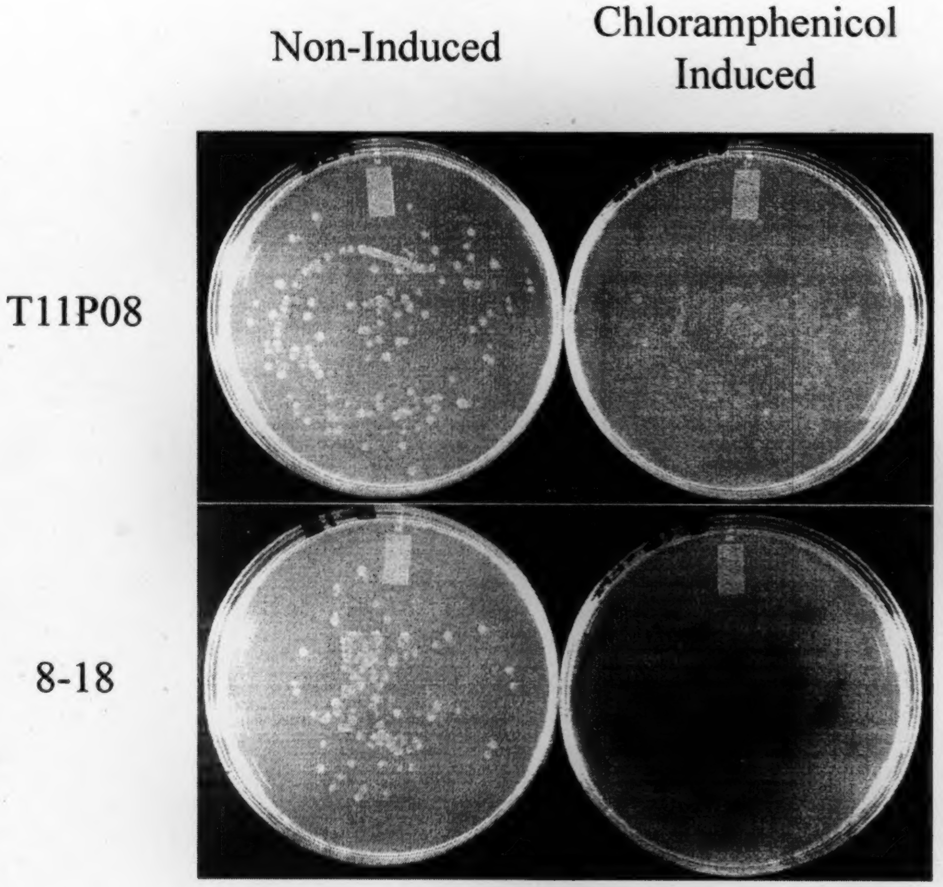

Non-Induced
Chloramphenicol Induced
cot-1
KCN
KCN
SHAM
cpk
KCN
KCN
SHAM
mcb
KCN
KCN
SHAM
os-1
KCN
KCN
SHAM
cr-1
KCN
KCN
SHAM

in the *aod-1* structural gene. In an attempt to avoid this predisposition, I chose to develop a reporter system that would exclude the isolation of structural gene mutations. Several earlier attempts to use reporter systems in the lab involved the reporter enzymes β-Gal, GFP, and GUS but were unsuccessful (Tanton and Nargang, personal communication; see sections 1.9 and 1.11). I decided to try the tyrosinase reporter system since it involves the use of an enzyme endogenous to *N. crassa* (see section 1.12). Previously, this system had been successfully used in *N. crassa* to isolate signaling components involved in controlling the *ccg-1* gene, a regulator of entry into conidiation (Kothe and Free, 1998).

3.4 Initial reporter strain

The tyrosinase reporter construct pBAT, which contains 3.3 kbp of *aod-1* upstream sequence fused to the coding sequence for *T*, as well as a bleomycin resistance marker, was constructed (Fig. 5). Four *T* mutant strains were obtained from S. Free and upon growth of these strains, it was observed that T1P11P08 and T1P11P13 grew and conidiated better than T1P11 and T1P11P16 (data not shown). Therefore, these two strains were chosen as tyrosinase mutant strains for the creation of the reporter strain. I transformed pBAT into sphaeroplasts of strains T1P11P08 and T1P11P13. Transformants resistant to bleomycin were selected and screened for reporter activity using the tyrosinase plate assay (see section 2.27). If the 3.3 kbp *aod-1* upstream region contained the sequences required for regulation of *aod-1* expression, then tyrosinase expression should be induced in transformant strains by *aod-1* inducing conditions. When grown on medium that does not induce alternative oxidase, colonies of strains carrying the reporter should remain white following the addition of tyrosine, while colonies of these strains grown on inducing media should turn brown with the addition of tyrosine.

During the first three to five hours of the tyrosinase plate assay, colonies of strains 8-1, 8-17, 8-18, and 8-19 from the transformation of T1P11P08 with pBAT, and colonies of strains 13-12, 13-13 and 13-16 from the transformation of T1P11P13 with pBAT, stayed white when grown on non-inducing media and turned brown when grown in the presence of chloramphenicol (Fig. 9). During the same time period, colonies of strains T1P11P08 and T1P11P13 remained white under both growth conditions (Fig. 9). However, after overnight incubation with the tyrosine solution, both the *T* mutant strains


Non-Induced
Chloramphenicol Induced
T11P08
8-18

and the reporter strains gave inconsistent results. Occasionally, colonies that were white after three to five hours of incubation with the tyrosine solution would remain white after 24 hours exposure, but more often would turn brown on all media. Nevertheless, it seemed that the colour difference observed during the first five hours of the assay would be sufficient to allow any of these strains to be used as a reporter strain. Southern analysis of these strains revealed that strain 8-18 contained a single copy of the reporter construct (data not shown) and this strain was chosen as the reporter strain for future work.

3.4.1 Mutagenesis of initial reporter strain

Several attempts to disrupt reporter expression with insertional mutagenesis were performed but were often confounded by technical difficulties with the tyrosinase plate assay and the reporter strain. I discovered that growth of sphaeroplast transformants within top agar and the presence of residual PMC solution, which is used in the transformation procedure, both prevented the colour change from occurring. The sphaeroplast transformation protocol was modified to remove the PMC and allow the sphaeroplasts to recover so that the cells could be spread on top of the plates. At this time, the lab began to use electroporation (Margolin *et al.*, 1997), and since electroporation does not involve PMC, this transformation method was found to be more compatible with the tyrosinase plate assay.

Approximately 13, 000 transformants from insertional mutagenesis using both sphaeroplast transformation and electroporation were screened for reporter expression. Some of these transformants were plated on non-inducing media to look for strains that constitutively produced tyrosinase, some on inducing media containing chloramphenicol to look for mutants that were unable to produce tyrosinase, and some on both non-inducing and inducing media. From these initial screens, a total of 152 putative mutant strains were obtained. 135 of these strains had constitutive tyrosinase activity, while 17 were unable to induce tyrosinase activity when grown in the presence of chloramphenicol. Each of the potential mutant strains was grown in non-inducing and inducing conditions, and re-tested with the tyrosinase plate assay. Of the original 152 potential mutants, 40 strains showed the same inappropriate expression of tyrosinase. Of these 40 strains, 32 turned brown during the plate assay in both conditions and 8 remained white in both conditions.

It was possible that strains unable to induce the reporter correctly contained mutations in the reporter construct itself. To rule out this possibility, I took advantage of the fact that the integration of the reporter construct was at an ectopic location in the genome, leaving the endogenous *aod-1* gene intact. If the insertional mutagenesis disrupted the regulation of reporter expression via an effect on a signaling pathway or a *trans*-acting factor that normally binds to the sequence upstream of the *aod-1* gene to affect transcription, then induction of *aod-1* itself should be affected similarly in these strains. Thus, strains that constitutively express the reporter would be expected to also constitutively express *aod-1*, while strains that were unable to induce the reporter should also be unable to induce *aod-1*. Respiration assays on these 40 strains grown in both alterative oxidase inducing and non-inducing conditions revealed that alternative oxidase activity was completely normal. Thus, despite the encouraging initial results, no alternative oxidase regulatory mutants were obtained.

3.5 Creation of oxidative phosphorylation mutant reporter strain

Since mutagenesis of the reporter strain was unsuccessful, I began to question the effectiveness of the selection scheme in isolating regulatory mutations. In an attempt to test whether or not the tyrosinase reporter system could be successful, I created a reporter strain that should constitutively induce both *aod-1* expression and expression of the reporter. Strain NCN5 carries a *cya-5* mutation that causes a cytochrome aa_3 deficiency and loss of complex IV function (Coffin *et al.*, 1997). Because of this disruption of the cytochrome pathway, alternative oxidase activity is always present in this strain.

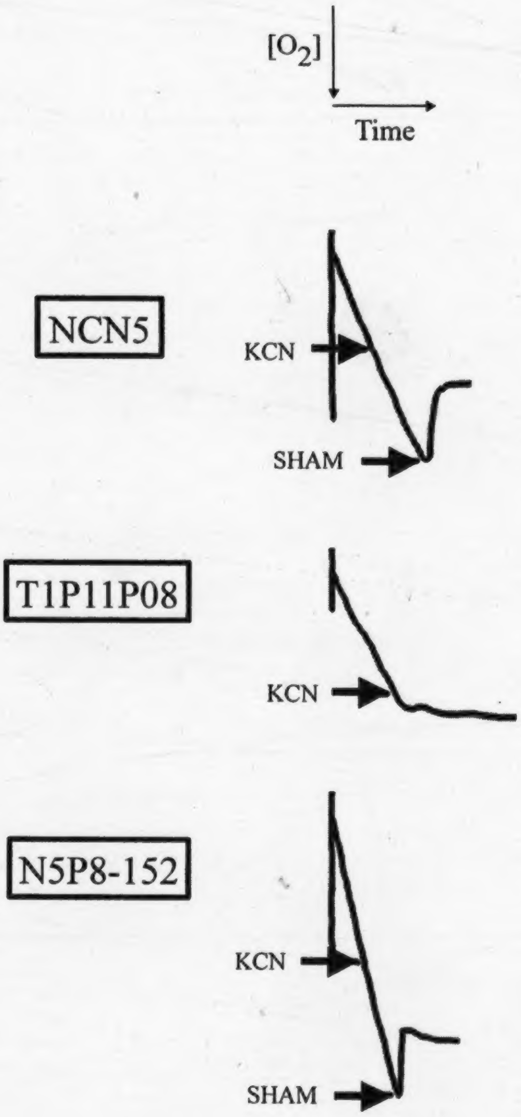
I crossed strains NCN5 (*cya-5*; *A*) with T1P11P08 (*T*, *al-2*, *a*) and screened the progeny for the desired phenotypes. Offspring that carried the *cya-5* mutation were identified through their constitutive cyanide-insensitive respiration. Albino (*al-2*) offspring were selected because this phenotype facilitates the observation of the colour change in the plate assay. The *al-2* locus is also linked (with 12% recombination) to the *T* locus on linkage group I, so albino progeny were most likely to be tyrosinase mutants as well (Horowitz and Fling, 1956).

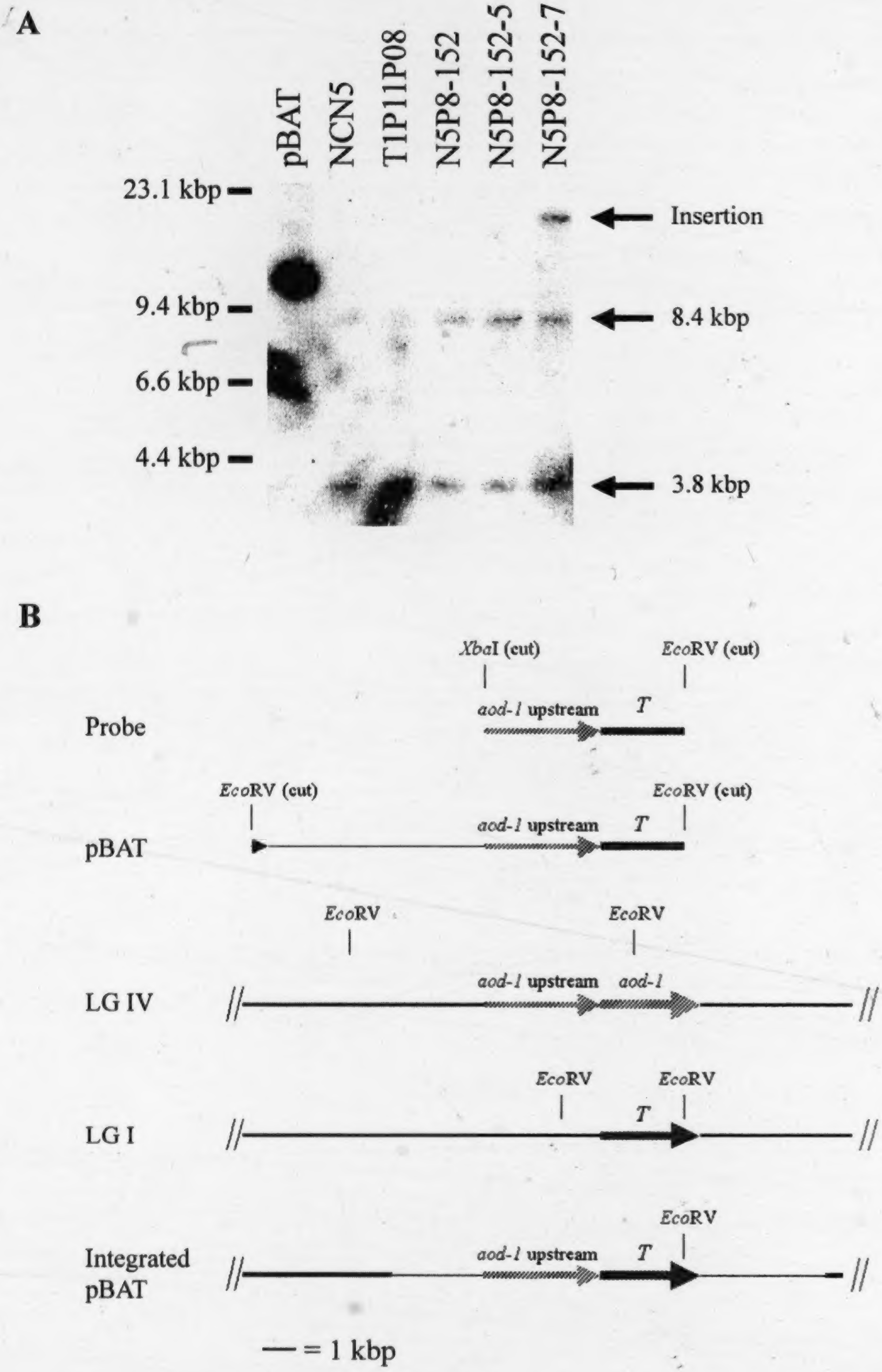
Strain N5P8-152 was selected as an albino strain that failed to turn brown during the tyrosinase plate assay, suggesting the presence of the mutant *T* allele. This strain also had cyanide-insensitive respiration when grown under non-inducing conditions, indicating the presence of the *cya-5* mutation (Fig. 10). I transformed strain N5P8-152 with the reporter construct pBAT to create the desired constitutively induced reporter strain. Transformants were selected on bleomycin and examined for their tyrosinase phenotype. Strains N5P8-152-5, and -7 grown on non-inducing media turned brown after only 1 hour in the tyrosinase plate assay. Southern analysis showed that strain N5P8-152-7 contained a single copy of the reporter (Fig. 11).

It was expected that if the reporter system functioned as predicted, colonies of strains that constitutively express *aod-1* would also constitutively express the reporter, and turn brown even more quickly than the original reporter strain. Since this was observed, it seemed that the selection scheme for isolation of regulatory mutations was sound. Generation of regulatory mutations that cause constitutive *aod-1* expression should similarly cause a rapid and reproducible colour change during the plate assay. However, no constitutive mutations had been successfully isolated to this point.

3.6 Characterization of the tyrosinase gene in mutant strains

Since the reporter system seemed to be functioning as predicted, I examined the tyrosinase mutant strains in an attempt to explain the lack of success at isolating mutants. Initial characterizations of potential reporter and mutant strains with the tyrosinase plate assay were difficult because comparison of the colour change that occurred during different tyrosinase plate assays for the same strains often gave conflicting results (see section 3.4). When subjected to the tyrosinase plate assay, the tyrosinase mutant strains T1P11P08 and T1P11P13 themselves displayed variable behavior and were either found to remain white or to develop the brown pigment over time. I originally attributed this variable colour change to leaky expression from the mutant *T* gene and tried to optimize conditions to prevent this colour change from occurring, including using fresh conidia and preventing the colonies on the plates from being over crowded. Regardless, the tyrosinase plate assay often seemed unreliable.


[O2]
Time
NCN5
KCN
SHAM
T1P11P08
KCN
N5P8-152
KCN
SHAM


A
pBAT
NCN5
T1P11P08
N5P8-152
N5P8-152-5
N5P8-152-7
23.1 kbp
9.4 kbp
6.6 kbp
4.4 kbp
Insertion
8.4 kbp
3.8 kbp
B
XbaI (cut)
EcoRV (cut)
Probe
aod-1 upstream
T
EcoRV (cut)
EcoRV (cut)
pBAT
aod-1 upstream
T
EcoRV
EcoRV
LG IV
aod-1 upstream
aod-1
EcoRV
EcoRV
LG I
T
EcoRV
Integrated pBAT
aod-1 upstream
T
— = 1 kbp

The lack of success using the original reporter strain prompted an interest in understanding the nature of the tyrosinase mutation in strains T1P11, T1P11P08 and T1P11P13. Therefore, I obtained copies of the *T* gene from the wild-type strain NCN235 and strain T1P11P08 through PCR, and subjected the PCR products to restriction digests. Since RIP causes transition mutations (Selker, 1990), I expected that the digestion patterns for some restriction enzymes would be altered. However, no difference in restriction patterns was observed (data not shown).

To examine the nature of the *T* alleles more closely, PCR products of the *T* gene in strains T1P11, T1P11P08 and T1P11P13 were obtained and sequenced. The T1P11P08 and T1P11P13 *T* sequences were identical, and both differed from that of strain T1P11. When compared to the published sequences (Kupper *et al.*, 1989) for the *T* gene from wild-type strains OR (74-OR8-1*a*; FGSC #988) and TS (69-1113; FGSC #2963), and later to the *T* gene sequence from the Whitehead database of wild-type strain 74-OR23-1V*A* (FGSC #2489; assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003), only strain T1P11 showed evidence of RIP transition mutations (Fig. 12). Thus, (much to my chagrin) strains T1P11P08 and T1P11P13 were not *T* mutants, explaining the lack of success using these strains.

The previous sequencing (Kupper *et al.*, 1989) of wild-type strains TS and OR revealed two different alleles of the *T* gene sequence, differing by 12 nucleotides between the TATA box and the polyadenylation signal. The region that I sequenced contains eight of these polymorphic sites. Strain T1P11 carries the same nucleotide as strain TS at 3 of these sites, and the same nucleotide as strain OR at the other 5. Strains T1P11P08 and T1P11P13, and the *T* sequence from the Whitehead database carry the same nucleotide as strain TS at only one site, while the other 7 nucleotides are identical to the OR sequence.

Interestingly, strains T1P11, T1P11P08, T1P11P13, and the Whitehead sequence all contain a novel polymorphic base at +1327, and a single nucleotide insertion at +2248 (with numbering starting at the initial methionine codon; Fig. 12). The altered nucleotide at +1327 does not change the amino acid sequence encoded by that codon. The insertion at +2248 occurs after the cleavage site for tyrosinase maturation (Kupper *et al.*, 1989) and changes the reading frame, replacing the last 26 codons reported for tyrosinase from strains OR and TS with 90 novel amino acids (Fig. 13). A previously developed

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                                                      ◆
T1P11       ................................TGTACCCACCACTCCCTCCAGCA   55
T1P11P08    .....................GCCATCACGGGTGTACCCACCCCTCCCTCCAGCA   55
OR          ATGAGCACCGACATCAAATTTGCCATCACGGGTGTACCCACCCCTCCCTCCAGCA   55
TS          ATGAGCACCGACATCAAATTTGCCATCACGGGTGTACCCACCACTCCCTCCAGCA   55
WHITEHEAD   ATGAGCACCGACATCAAATTTGCCATCACGGGTGTACCCACCCCTCCCTCCAGCA   55

T1P11       ATGgtgaccctcccttcccttcgttataactgcttgtactaacacaaacacccag  110
T1P11P08    ATGgtgaccctcccttcccttcgttatgactgcttgtactaacacaaacacccag  110
OR          ATGgtgaccctcccttcccttcgttatgactgcttgtactaacacaaacacccag  110
TS          ATGgtgaccctcccttcccttcgttatgactgcttgtactaacacaaacacccag  110
WHITEHEAD   ATGgtgaccctcccttcccttcgttatgactgcttgtactaacacaaacacccag  110

T1P11       GAGCGGTCCCTCTCCGCCGCGAGCTCCGCGACCTCCAACAAAACTACCCGGAGCA  165
T1P11P08    GAGCGGTCCCTCTCCGCCGCGAGCTCCGCGACCTCCAACAAAACTACCCGGAGCA  165
OR          GAGCGGTCCCTCTCCGCCGCGAGCTCCGCGACCTCCAACAAAACTACCCGGAGCA  165
TS          GAGCGGTCCCTCTCCGCCGCGAGCTCCGCGACCTCCAACAAAACTACCCGGAGCA  165
WHITEHEAD   GAGCGGTCCCTCTCCGCCGCGAGCTCCGCGACCTCCAACAAAACTACCCGGAGCA  165

T1P11       GTTCAATCTCTACCTCCTCGGCCTGCGCGACTTCCAAGGCCTCGACGAAGCCAAA  220
T1P11P08    GTTCAATCTCTACCTCCTCGGCCTGCGCGACTTCCAAGGCCTCGACGAAGCCAAG  220
OR          GTTCAATCTCTACCTCCTCGGCCTGCGCGACTTCCAAGGCCTCGACGAAGCCAAG  220
TS          GTTCAATCTCTACCTCCTCGGCCTGCGCGACTTCCAAGGCCTCGACGAAGCCAAG  220
WHITEHEAD   GTTCAATCTCTACCTCCTCGGCCTGCGCGACTTCCAAGGCCTCGACGAAGCCAAG  220
                                             ◆     ◆
T1P11       CTAAACTCGTACTACCAAATCGCTGGCATTCACGGTATGCCCTTCAAGCCGTAGG  275
T1P11P08    CTAGACTCGTACTACCAAGTCGCTGGCATTCACGGTATGCCCTTCAAGCCGTGGG  275
OR          CTAGACTCGTACTACCAAGTCGCTGGCATTCACGGTATGCCCTTCAAGCCGTGGG  275
TS          CTAGACTCGTACTACCAAGTCGCTGGCATCCACGGCATGCCCTTCAAGCCGTGGG  275
WHITEHEAD   CTAGACTCGTACTACCAAGTCGCTGGCATTCACGGTATGCCCTTCAAGCCGTGGG  275

T1P11       CCGGTGTCCCTTCCGACACGGACTGGTCCCAACCCGGCAGCAGCGGCTTTAGCGG  330
T1P11P08    CCGGTGTCCCTTCCGACACGGACTGGTCCCAACCCGGCAGCAGCGGCTTTGGCGG  330
OR          CCGGTGTCCCTTCCGACACGGACTGGTCCCAACCCGGCAGCAGCGGCTTTGGCGG  330
TS          CCGGTGTCCCTTCCGACACGGACTGGTCCCAACCCGGCAGCAGCGGCTTTGGCGG  330
WHITEHEAD   CCGGTGTCCCTTCCGACACGGACTGGTCCCAACCCGGCAGCAGCGGCTTTGGCGG  330

T1P11       CTACTGCACGCACTCGTCCATCCTCTTCATCACCTGGCACAGGCCCTACCTTGCT  385
T1P11P08    CTACTGCACGCACTCGTCCATCCTCTTCATCACCTGGCACAGGCCCTACCTTGCT  385
OR          CTACTGCACGCACTCGTCCATCCTCTTCATCACCTGGCACAGGCCCTACCTTGCT  385
TS          CTACTGCACGCACTCGTCCATCCTCTTCATCACCTGGCACAGGCCCTACCTTGCT  385
WHITEHEAD   CTACTGCACGCACTCGTCCATCCTCTTCATCACCTGGCACAGGCCCTACCTTGCT  385

T1P11       CTGTACGAGCAGGCCCTCTACGCCTCCGTACAAGCCGTTGCCCAAAAGTTCCCTG  440
T1P11P08    CTGTACGAGCAGGCCCTCTACGCCTCCGTGCAAGCCGTTGCCCAAAAGTTCCCTG  440
OR          CTGTACGAGCAGGCCCTCTACGCCTCCGTGCAAGCCGTTGCCCAAAAGTTCCCTG  440
TS          CTGTACGAGCAGGCCCTCTACGCCTCCGTGCAAGCCGTTGCCCAAAAGTTCCCTG  440
WHITEHEAD   CTGTACGAGCAGGCCCTCTACGCCTCCGTGCAAGCCGTTGCCCAAAAGTTCCCTG  440

T1P11       TCGAAGGGGGGCTGAGAGCCAAGTATGTCGCGGCCGCCAAGGATTTCAGGGCGCC  495
T1P11P08    TCGAAGGGGGGCTGAGAGCCAAGTATGTCGCGGCCGCCAAGGATTTCAGGGCGCC  495
OR          TCGAAGGGGGGCTGAGAGCCAAGTATGTCGCGGCCGCCAAGGATTTCAGGGCGCC  495
TS          TCGAAGGGGGGCTGAGAGCCAAGTATGTCGCGGCCGCCAAGGATTTCAGGGCGCC  495
WHITEHEAD   TCGAAGGGGGGCTGAGAGCCAAGTATGTCGCGGCCGCCAAGGATTTCAGGGCGCC  495
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T1P11      GTACTTTGATTGGGCTAGTCAACCTCCTAAAGGAACGCTGGCGTTTCCGGAGTCC  550
T1P11P08   GTACTTTGATTGGGCTAGTCAGCCTCCTAAAGGAACGCTGGCGTTTCCGGAGTCC  550
OR         GTACTTTGATTGGGCTAGTCAGCCTCCTAAAGGAACGCTGGCGTTTCCGGAGTCC  550
TS         GTACTTTGATTGGGCTAGTCAGCCTCCTAAAGGAACGCTGGCGTTTCCGGAGTCC  550
WHITEHEAD  GTACTTTGATTGGGCTAGTCAGCCTCCTAAAGGAACGCTGGCGTTTCCGGAGTCC  550

T1P11      CTCTCATCGCGTACCATACAAGTGGTGGATATCAACAGGAAGACCAAGAGCATCA  605
T1P11P08   CTCTCATCGCGTACCATACAGGTGGTGGATGTCGACGGGAAGACCAAGAGCATCA  605
OR         CTCTCATCGCGTACCATACAGGTGGTGGATGTCGACGGGAAGACCAAGAGCATCA  605
TS         CTCTCATCGCGTACCATACAGGTGGTGGATGTCGACGGGAAGACCAAGAGCATCA  605
WHITEHEAD  CTCTCATCGCGTACCATACAGGTGGTGGATGTCGACGGGAAGACCAAGAGCATCA  605

T1P11      ACAACCCGCTACACCAATTTACTTTCCACCCGGTCAACCCTAGCCCTGGTAACTT  660
T1P11P08   ACAACCCGCTGCACCGGTTTACTTTCCACCCGGTCAACCCTAGCCCTGGTGACTT  660
OR         ACAACCCGCTGCACCGGTTTACTTTCCACCCGGTCAACCCTAGCCCTGGTGACTT  660
TS         ACAACCCGCTGCACCGGTTTACTTTCCACCCGGTCAACCCTAGCCCTGGTGACTT  660
WHITEHEAD  ACAACCCGCTGCACCGGTTTACTTTCCACCCGGTCAACCCTAGCCCTGGTGACTT  660

T1P11      CAGCGCGGCGTGGAGCAAATACCCAAGCACGGTCCGGTATCCAAACCGGCTGACA  715
T1P11P08   CAGCGCGGCGTGGAGCAGATACCCAAGCACGGTCCGGTATCCAAACCGGCTGACA  715
OR         CAGCGCGGCGTGGAGCAGATACCCAAGCACGGTCCGGTATCCAAACCGGCTGACA  715
TS         CAGCGCGGCGTGGAGCAGATACCCAAGCACGGTCCGGTATCCAAACCGGCTGACA  715
WHITEHEAD  CAGCGCGGCGTGGAGCAGATACCCAAGCACGGTCCGGTATCCAAACCGGCTGACA  715

T1P11      GGGGCATCGCGGATGAGCGGATTGCGCCCATCTTGGCGAACAAACTAGCGTCAT  770
T1P11P08   GGGGCATCGCGGATGAGCGGATTGCGCCCATCTTGGCGAACGAGCTGGCGTCAT  770
OR         GGGGCATCGCGGATGAGCGGATTGCGCCCATCTTGGCGAACGAGCTGGCGTCAT  770
TS         GGGGCATCGCGGATGAGCGGATTGCGCCCATCTTGGCGAACGAGCTGGCGTCAT  770
WHITEHEAD  GGGGCATCGCGGATGAGCGGATTGCGCCCATCTTGGCGAACGAGCTGGCGTCAT  770

T1P11      TAAGAAATAATATCAATTTGTTGTTGCTGAGCTACAAGGACTTTGATGCGTTCAG  825
T1P11P08   TGAGAAATAATGTCAGTTTGTTGTTGCTGAGCTACAAGGACTTTGATGCGTTCAG  825
OR         TGAGAAATAATGTCAGTTTGTTGTTGCTGAGCTACAAGGACTTTGATGCGTTCAG  825
TS         TGAGAAATAATGTCAGTTTGTTGTTGCTGAGCTACAAGGACTTTGATGCGTTCAG  825
WHITEHEAD  TGAGAAATAATGTCAGTTTGTTGTTGCTGAGCTACAAGGACTTTGATGCGTTCAG  825
                                                     ◆
T1P11      TTATAACAGGTGGGATCCGAATACCAACCCGGGTAATTTCGGCAATTTGGAAGAT  880
T1P11P08   TTATAACAGGTGGGATCCGAATACCAACCCGGGTGATTTCGGCAGTTTGGAGGAT  880
OR         TTATAACAGGTGGGATCCGAATACCAACCCGGGTGATTTCGGTAGTTTGGAGGAT  880
TS         TTATAACAGGTGGGATCCGAATACCAACCCGGGTGATTTCGGCAGTTTGGAGGAT  880
WHITEHEAD  TTATAACAGGTGGGATCCGAATACCAACCCGGGTGATTTCGGCAGTTTGGAGGAT  880

T1P11      ATGCATAATGAGATTCATAATCGGACAGGCGGAAATGGACATATGTCGAGCCTGG  935
T1P11P08   GTGCATAATGAGATTCATGATCGGACAGGCGGAAATGGACATATGTCGAGCCTGG  935
OR         GTGCATAATGAGATTCATGATCGGACAGGCGGAAATGGACATATGTCGAGCCTGG  935
TS         GTGCATAATGAGATTCATGATCGGACAGGCGGAAATGGACATATGTCGAGCCTGG  935
WHITEHEAD  GTGCATAATGAGATTCATGATCGGACAGGCGGAAATGGACATATGTCGAGCCTGG  935

T1P11      AGGTGTCGGCTTTTGATCCGCTGTTTTGGTTGCACCATGTgtatgtggtcactcc  990
T1P11P08   AGGTGTCGGCTTTTGATCCGCTGTTTTGGTTGCACCATGTgtatgtggtcactcc  990
OR         AGGTGTCGGCTTTTGATCCGCTGTTTTGGTTGCACCATGTgtatgtggtcactcc  990
TS         AGGTGTCGGCTTTTGATCCGCTGTTTTGGTTGCACCATGTgtatgtggtcactcc  990
WHITEHEAD  AGGTGTCGGCTTTTGATCCGCTGTTTTGGTTGCACCATGTgtatgtggtcactcc  990
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T1P11      ccttttcccttttttctttgcatcggtcatgcctgacaggccccattccgttatc 1045
T1P11P08   ccttttcccttttttctttgcatcggtcatgcctgacaggccccattccgttatc 1045
OR         ccttttcccttttttctttgcatcggtcatgcctgacaggccccattccgttatc 1045
TS         ccttttcccttttttctttgcatcggtcatgcctgacaggccccattccgttatc 1045
WHITEHEAD  ccttttcccttttttctttgcatcggtcatgcctgacaggccccattccgttatc 1045

T1P11      gtcgctgacaagaagcccttgatctgaagTAACGTCGACCGCCTCTGGTCTATCT 1100
T1P11P08   gtcgctgacaagaagcccttgatctgaagTAACGTCGACCGCCTCTGGTCTATCT 1100
OR         gtcgctgacaagaagcccttgatctgaagTAACGTCGACCGCCTCTGGTCTATCT 1100
TS         gtcgctgacaagaagcccttgatctgaagTAACGTCGACCGCCTCTGGTCTATCT 1100
WHITEHEAD  gtcgctgacaagaagcccttgatctgaagTAACGTCGACCGCCTCTGGTCTATCT 1100

T1P11      GGCAAGACCTTAACCCCAACAACTTCATAACTCCCCGACCCGCTCCCTACTCGAC 1155
T1P11P08   GGCAAGACCTTAACCCCAACAGCTTCATGACTCCCCGACCCGCTCCCTACTCGAC 1155
OR         GGCAAGACCTTAACCCCAACAGCTTCATGACTCCCCGACCCGCTCCCTACTCGAC 1155
TS         GGCAAGACCTTAACCCCAACAGCTTCATGACTCCCCGACCCGCTCCCTACTCGAC 1155
WHITEHEAD  GGCAAGACCTTAACCCCAACAGCTTCATGACTCCCCGACCCGCTCCCTACTCGAC 1155

T1P11      CTTCGTCGCACAGGAGGGTAAAAGCCAAAGTAAGAACACACCTCTAGAGCCGTTC 1210
T1P11P08   CTTCGTCGCACAGGAGGGTGAAAGCCAGAGTAAGAGCACGCCTCTGGAGCCGTTC 1210
OR         CTTCGTCGCACAGGAGGGTGAAAGCCAGAGTAAGAGCACGCCTCTGGAGCCGTTC 1210
TS         CTTCGTCGCACAGGAGGGTGAAAGCCAGAGTAAGAGCACGCCTCTGGAGCCGTTC 1210
WHITEHEAD  CTTCGTCGCACAGGAGGGTGAAAGCCAGAGTAAGAGCACGCCTCTGGAGCCGTTC 1210

T1P11      TAGGACAAATCTACTGCCAACTTTTGGACCTCTAAGCAGGTCAAGGACAGCATCA 1265
T1P11P08   TGGGACAAATCTGCTGCCAACTTTTGGACCTCTGAGCAGGTCAAGGACAGCATCA 1265
OR         TGGGACAAATCTGCTGCCAACTTTTGGACCTCTGAGCAGGTCAAGGACAGCATCA 1265
TS         TGGGACAAATCTGCTGCCAACTTTTGGACCTCTGAGCAGGTCAAGGACAGCATCA 1265
WHITEHEAD  TGGGACAAATCTGCTGCCAACTTTTGGACCTCTGAGCAGGTCAAGGACAGCATCA 1265

T1P11      CCTTCGGATACGCGTATCCTAAGACGCAAAAGTGGAAGTACTCGTCCGTAAAGGA 1320
T1P11P08   CCTTCGGATACGCGTATCCTGAGACGCAGAAGTGGAAGTACTCGTCCGTAAAGGA 1320
OR         CCTTCGGATACGCGTATCCTGAGACGCAGAAGTGGAAGTACTCGTCCGTAAAGGA 1320
TS         CCTTCGGATACGCGTATCCTGAGACGCAGAAGTGGAAGTACTCGTCCGTAAAGGA 1320
WHITEHEAD  CCTTCGGATACGCGTATCCTGAGACGCAGAAGTGGAAGTACTCGTCCGTAAAGGA 1320
                 ●                                                ▼
T1P11      GTACCAGGCAGCCATCAGGAAGTCTGTCACGGCGCTATACGGGAGCAATGTGTTT 1375
T1P11P08   GTACCAGGCAGCCATCAGGAAGTCTGTCACGGCGCTGTACGGGAGCAATGTGTTT 1375
OR         GTACCAAGCAGCCATCAGGAAGTCTGTCACGGCGCTGTACGGGAGCAATGTGTTT 1375
TS         GTACCAAGCAGCCATCAGGAAGTCTGTCACGGCGCTGTACGGGAGCAATGTGTTT 1375
WHITEHEAD  GTACCAGGCAGCCATCAGGAAGTCTGTCACGGCGCTGTACGGGAGCAATGTGTTT 1375
                                                          ◆
T1P11      ACCAACTTTATTAAGAATATCGCCGACCGAACGCCGGCGTTGAAGAACCCGCAGG 1430
T1P11P08   GCCAACTTTGTTGAGAATGTCGCCGACCGAACGCCGGCGTTGAAGAAACCGCAGG 1430
OR         GCCAACTTTGTTGAGAATGTCGCCGACCGAACGCCGGCGTTGAAGAAACCGCAGG 1430
TS         GCCAACTTTGTTGAGAATGTCGCCGACCGAACGCCGGCGTTGAAGAACCCGCAGG 1430
WHITEHEAD  GCCAACTTTGTTGAGAATGTCGCCGACCGAACGCCGGCGTTGAAGAAACCGCAGG 1430

T1P11      CAACTGGCGAGGAGAGCAAGAGCACCGTCTCGGCTGCTGCTGCTCATGCAATTGA 1485
T1P11P08   CAACTGGCGAGGAGAGCAAGAGCACCGTCTCGGCTGCTGCTGCTCATGCAGTTGA 1485
OR         CAACTGGCGAGGAGAGCAAGAGCACCGTCTCGGCTGCTGCTGCTCATGCAGTTGA 1485
TS         CAACTGGCGAGGAGAGCAAGAGCACCGTCTCGGCTGCTGCTGCTCATGCAGTTGA 1485
WHITEHEAD  CAACTGGCGAGGAGAGCAAGAGCACCGTCTCGGCTGCTGCTGCTCATGCAGTTGA 1485
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                          ◆
T1P11      GCTTTCCGGGGCCAAAAAGGTCGCAGAGAAGGTCCATAATGTATTCCAGCACGCT 1540
T1P11P08   GCTTTCCGGGGCCAAGAAGGTCGCAGAGAAGGTCCATAATGTGTTCCAGCACGCT 1540
OR         GCTTTCCGGGGCCAAGAAGGTCGCAGAGAAGGTCCATAATGTGTTCCAGCACGCT 1540
TS         GCTTTCCGGGGCCAGGAAGGTCGCAGAGAAGGTCCATAATGTGTTCCAGCACGCT 1540
WHITEHEAD  GCTTTCCGGGGCCAAGAAGGTCGCAGAGAAGGTCCATAATGTGTTCCAGCACGCT 1540

T1P11      AAGGAGAAAGCGCAAAAGCCGGTAGTACCTATTAAGGATACAAAGGCCGAATCGA 1595
T1P11P08   GAGGAGAAGGCGCAGAAGCCGGTGGTGCCTGTTAAGGATACAAAGGCCGAGTCGA 1595
OR         GAGGAGAAGGCGCAGAAGCCGGTGGTGCCTGTTAAGGATACAAAGGCCGAGTCGA 1595
TS         GAGGAGAAGGCGCAGAAGCCGGTGGTGCCTGTTAAGGATACAAAGGCCGAGTCGA 1595
WHITEHEAD  GAGGAGAAGGCGCAGAAGCCGGTGGTGCCTGTTAAGGATACAAAGGCCGAGTCGA 1595

T1P11      GCACGGCCGCAAGTATAATGATCGGCTTGAGCATAAAACGTCCATCCAAACTAAC 1650
T1P11P08   GCACGGCCGCAGGTATGATGATCGGCTTGAGCATAAAACGTCCATCCAAGCTGAC 1650
OR         GCACGGCCGCAGGTATGATGATCGGCTTGAGCATAAAACGTCCATCCAAGCTGAC 1650
TS         GCACGGCCGCAGGTATGATGATCGGCTTGAGCATAAAACGTCCATCCAAGCTGAC 1650
WHITEHEAD  GCACGGCCGCAGGTATGATGATCGGCTTGAGCATAAAACGTCCATCCAAGCTGAC 1650

T1P11      AGCTTCACCAGGCCCAATTCCAGAGAGCCTCAAGTACCTCGCCCCAAACGGAAAG 1705
T1P11P08   AGCTTCACCAGGCCCAATTCCAGAGAGCCTCAAGTACCTCGCCCCAGACGGAAAG 1705
OR         AGCTTCACCAGGCCCAATTCCAGAGAGCCTCAAGTACCTCGCCCCAGACGGAAAG 1705
TS         AGCTTCACCAGGCCCAATTCCAGAGAGCCTCAAGTACCTCGCCCCAGACGGAAAG 1705
WHITEHEAD  AGCTTCACCAGGCCCAATTCCAGAGAGCCTCAAGTACCTCGCCCCAGACGGAAAG 1705

T1P11      TACACCGACTGGATTGTCAACGTTCGCGCCCAAAAACACGGTCTAGGGCAATCCT 1760
T1P11P08   TACACCGACTGGATTGTCAACGTTCGCGCCCAGAAACACGGTCTGGGGCAATCCT 1760
OR         TACACCGACTGGATTGTCAACGTTCGCGCCCAGAAACACGGTCTGGGGCAATCCT 1760
TS         TACACCGACTGGATTGTCAACGTTCGCGCCCAGAAACACGGTCTGGGGCAATCCT 1760
WHITEHEAD  TACACCGACTGGATTGTCAACGTTCGCGCCCAGAAACACGGTCTGGGGCAATCCT 1760

T1P11      TCCGTATCATCGTCTTCTTAGGCGAATTCAACCCCGACCCAGAGACCTAGAACGA 1815
T1P11P08   TCCGTGTCATCGTCTTCTTGGGCGAGTTCAACCCCGACCCAGAGACCTGGGACGA 1815
OR         TCCGTGTCATCGTCTTCTTGGGCGAGTTCAACCCCGACCCAGAGACCTGGGACGA 1815
TS         TCCGTGTCATCGTCTTCTTGGGCGAGTTCAACCCCGACCCAGAGACCTGGGACGA 1815
WHITEHEAD  TCCGTGTCATCGTCTTCTTGGGCGAGTTCAACCCCGACCCAGAGACCTGGGACGA 1815

T1P11      CGAGTTCAACTGCGTCGGTCGTGTGTCCGTATTGGGACGGAGCGCCGAAACCCAG 1870
T1P11P08   CGAGTTCAACTGCGTCGGTCGTGTGTCCGTGTTGGGACGGAGCGCCGAAACCCAG 1870
OR         CGAGTTCAACTGCGTCGGTCGTGTGTCCGTGTTGGGACGGAGCGCCGAAACCCAG 1870
TS         CGAGTTCAACTGCGTCGGTCGTGTGTCCGTGTTGGGACGGAGCGCCGAAACCCAG 1870
WHITEHEAD  CGAGTTCAACTGCGTCGGTCGTGTGTCCGTGTTGGGACGGAGCGCCGAAACCCAG 1870

T1P11      TGTAGCAAGTGCCGCAAGGATAACGCAAACGGTCTAATCGTCTCAAGCACTATGC 1925
T1P11P08   TGTGGCAAGTGCCGCAAGGATAACGCAAACGGTCTGATCGTCTCAGGCACTGTGC 1925
OR         TGTGGCAAGTGCCGCAAGGATAACGCAAACGGTCTGATCGTCTCAGGCACTGTGC 1925
TS         TGTGGCAAGTGCCGCAAGGATAACGCAAACGGTCTGATCGTCTCAGGCACTGTGC 1925
WHITEHEAD  TGTGGCAAGTGCCGCAAGGATAACGCAAACGGTCTGATCGTCTCAGGCACTGTGC 1925
                     ●
T1P11      CCCTAACCTCCGCTTTACTGCAGGATATTGTAGGCGGCGAGCTCCAGAGCCTCAA 1980
T1P11P08   CCCTGACCTCCGCTTTGCTGCAGGATATTGTGGGCGGCGAGCTCCAGAGCCTCAA 1980
OR         CCCTGACCTC.GCTTTGCTGCAGGATATTGTGGGCGGCGAGCTCCAGAGCCTCAA 1979
TS         CCCTGACCTC.GCTTTGCTGCAGGATATTGTGGGCGGCGAGCTCCAGAGCCTCAA 1979
WHITEHEAD  CCCTGACCTCCGCTTTGCTGCAGGATATTGTGGGCGGCGAGCTCCAGAGCCTCAA 1980
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T1P11      GCCTAAGGATGTCATCCCGCATCTGCGCGCCAACTTGAAGTGGAAGGTAACCCTC 2035
T1P11P08   GCCTGAGGATGTCATCCCGCATCTGCGCGCCAACTTGAAGTGGAAGGTGGCCCTC 2035
OR         GCCTGAGGATGTCATCCCGCATCTGCGCGCCAACTTGAAGTGGAAGGTGGCCCTC 2034
TS         GCCTGAGGATGTCATCCCGCATCTGCGCGCCAACTTGAAGTGGAAGGTGGCCCTC 2034
WHITEHEAD  GCCTGAGGATGTCATCCCGCATCTGCGCGCCAACTTGAAGTGGAAGGTGGCCCTC 2035

T1P11      TTCAACGGCGACGAGTATAATCTAAAGGAGGTTCCGGACCTCAAGGTTAGTGTGG 2090
T1P11P08   TTCAACGGCGACGAGTATAATCTGGAGGAGGTTCCGGACCTCAAGGTTAGTGTGG 2090
OR         TTCAACGGCGACGAGTATAATCTGGAGGAGGTTCCGGACCTCAAGGTTAGTGTGG 2089
TS         TTCAACGGCGACGAGTATAATCTGGAGGAGGTTCCGGACCTCAAGGTTAGTGTGG 2089
WHITEHEAD  TTCAACGGCGACGAGTATAATCTGGAGGAGGTTCCGGACCTCAAGGTTAGTGTGG 2090

T1P11      CTTCAACAGAGGTGACTATCGATAAGGAAGGCTTACCACATTACTCTCGCCAATA 2145
T1P11P08   CTTCGACAGAGGTGACTATCGATGAGGAAGGCTTGCCACATTACTCTCGCCAGTA 2145
OR         CTTCGACAGAGGTGACTATCGATGAGGAAGGCTTGCCACATTACTCTCGCCAGTA 2144
TS         CTTCGACAGAGGTGACTATCGATGAGGAAGGCTTGCCACATTACTCTCGCCAGTA 2144
WHITEHEAD  CTTCGACAGAGGTGACTATCGATGAGGAAGGCTTGCCACATTACTCTCGCCAGTA 2145
                                                ◆
T1P11      TACCGTCTATCCTAAGATCACCGAGGGGAAGCCGTACGGACATGGCCCAGAGGAT 2200
T1P11P08   TACCGTCTATCCTGAGATCACCGAGGGGAAGCCGTGCGGACATGGCCCAGAGGAT 2200
OR         TACCGTCTATCCTGAGATCACCGAGGGGAAGCCGTGCGGACATGGCCCAGAGGAT 2199
TS         TACCGTCTATCCTGAGATCACCGAGGGGAAGCCGTGCCGACATGGCCCAGAGGAT 2199
WHITEHEAD  TACCGTCTATCCTGAGATCACCGAGGGGAAGCCGTGCGGACATGGCCCAGAGGAT 2200

T1P11      CACATCTAAAGGGGGATTCAGCATCAAAACGTAGGAAGGTCCAAAGGGGGGGGTC 2255
T1P11P08   CACATCTAAAGGGGGATTCAGCATCAGAACGTAGGAAGGTCCAAAGGGGGGGGTC 2255
OR         CACATCTAAAGGGGGATTCAGCATCAGAACGTAGGAAGGTCCAAAGGGGGGGGTC 2254
TS         CACATCTAAAGGGGGATTCAGCATCAGAACGTAGGAAGGTCCAAAGGGGGGGGTC 2254
WHITEHEAD  CACATCTAAAGGGGGATTCAGCATCAGAACGTAGGAAGGTCCAAAGGGGGGGGTC 2255
                                  ◆
T1P11      CTGTAGGGTGAAATCAAAAAACCGGGTA                            2283
T1P11P08   CTGTAGGGTGAGATCAAGAAACCGGGTA                            2283
OR         CTGTAGGGTGAGATCAAGAAACCGGGTA                            2282
TS         CTGTAGGGTGAGATCAAGAAACCCGGTA                            2282
WHITEHEAD  CTGTAGGGTGAGATCAAGAAACCGGGTA                            2283
```

x = Site of RIP base change

xxx = Start and stop codons

● = Newly identified polymorphic site

◆ = Previously identified polymorphic site, OR vs. TS (Kupper *et al.*, 1989)

↓ = Predicted cleavage site for tyrosinase maturation (Kupper *et al.*, 1989)

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           ◆
T1P11      ...........VPTSPSSNGAVPLRRELRDLQQNYPEQFNLYLLGLRDFQGLDEA   55
WHITEHEAD  MSTDIKFAITGVPTPPSSNGAVPLRRELRDLQQNYPEQFNLYLLGLRDFQGLDEA   55
OR         MSTDIKFAITGVPTPPSSNGAVPLRRELRDLQQNYPEQFNLYLLGLRDFQGLDEA   55
TS         MSTDIKFAITGVPTTPSSNGAVPLRRELRDLQQNYPEQFNLYLLGLRDFQGLDEA   55

                             ★                    ┌┐
T1P11      KLNSYYQIAGIHGMPFKP.AGVPSDTDWSQPGSSGFSGYCTHSSILFITWHRPYL  110
WHITEHEAD  KLDSYYQVAGIHGMPFKPWAGVPSDTDWSQPGSSGFGGYCTHSSILFITWHRPYL  110
OR         KLDSYYQVAGIHGMPFKPWAGVPSDTDWSQPGSSGFGGYCTHSSILFITWHRPYL  110
TS         KLDSYYQVAGIHGMPFKPWAGVPSDTDWSQPGSSGFGGYCTHSSILFITWHRPYL  110

T1P11      ALYEQALYASVQAVAQKFPVEGGLRAKYVAAAKDFRAPYFDWASQPPKGTLAFPE  165
WHITEHEAD  ALYEQALYASVQAVAQKFPVEGGLRAKYVAAAKDFRAPYFDWASQPPKGTLAFPE  165
OR         ALYEQALYASVQAVAQKFPVEGGLRAKYVAAAKDFRAPYFDWASQPPKGTLAFPE  165
TS         ALYEQALYASVQAVAQKFPVEGGLRAKYVAAAKDFRAPYFDWASQPPKGTLAFPE  165

T1P11      SLSSRTIQVVDINRKTKSINNPLHQFTFHPVNPSPGNFSAAWSKYPSTVRYPNRL  220
WHITEHEAD  SLSSRTIQVVDVDGKTKSINNPLHRFTFHPVNPSPGDFSAAWSRYPSTVRYPNRL  220
OR         SLSSRTIQVVDVDGKTKSINNPLHRFTFHPVNPSPGDFSAAWSRYPSTVRYPNRL  220
TS         SLSSRTIQVVDVDGKTKSINNPLHRFTFHPVNPSPGDFSAAWSRYPSTVRYPNRL  220

T1P11      TGASRDERIAPILANKLASLRNNINLLLLSYKDFDAFSYNRWDPNTNPGNFGNLE  275
WHITEHEAD  TGASRDERIAPILANELASLRNNVSLLLLSYKDFDAFSYNRWDPNTNPGDFGSLE  275
OR         TGASRDERIAPILANELASLRNNVSLLLLSYKDFDAFSYNRWDPNTNPGDFGSLE  275
TS         TGASRDERIAPILANELASLRNNVSLLLLSYKDFDAFSYNRWDPNTNPGDFGSLE  275

T1P11      DMHNEIHNRTGGNGHMSSLEVSAFDPLFWLHHVNVDRLWSIWQDLNPNNFITPRP  330
WHITEHEAD  DVHNEIHDRTGGNGHMSSLEVSAFDPLFWLHHVNVDRLWSIWQDLNPNSFMTPRP  330
OR         DVHNEIHDRTGGNGHMSSLEVSAFDPLFWLHHVNVDRLWSIWQDLNPNSFMTPRP  330
TS         DVHNEIHDRTGGNGHMSSLEVSAFDPLFWLHHVNVDRLWSIWQDLNPNSFMTPRP  330

                                  ★
T1P11      APYSTFVAQEGKSQSKNTPLEPF.DKSTANFWTSKQVKDSITFGYAYPKTQKWKY  385
WHITEHEAD  APYSTFVAQEGESQSKSTPLEPFWDKSAANFWTSEQVKDSITFGYAYPETQKWKY  385
OR         APYSTFVAQEGESQSKSTPLEPFWDKSAANFWTSEQVKDSITFGYAYPETQKWKY  385
TS         APYSTFVAQEGESQSKSTPLEPFWDKSAANFWTSEQVKDSITFGYAYPETQKWKY  385

                                  ●
T1P11      SSVKEYQAAIRKSVTALYGSNVFTNFIKNIADRTPALKNPQATGEESKSTVSAAA  440
WHITEHEAD  SSVKEYQAAIRKSVTALYGSNVFANFVENVADRTPALKKPQATGEESKSTVSAAA  440
OR         SSVKEYQAAIRKSVTALYGSNVFANFVENVADRTPALKKPQATGEESKSTVSAAA  440
TS         SSVKEYQAAIRKSVTALYGSNVFANFVENVADRTPALKNPQATGEESKSTVSAAA  440
                                  ●

T1P11      AHAIELSGAKKVAEKVHNVFQHAKEKAQKPVVPIKDTKAESSTAASIMIGLSIKR  495
WHITEHEAD  AHAVELSGAKKVAEKVHNVFQHAEEKAQKPVVPVKDTKAESSTAAGMMIGLSIKR  495
OR         AHAVELSGAKKVAEKVHNVFQHAEEKAQKPVVPVKDTKAESSTAAGMMIGLSIKR  495
TS         AHAVELSGARKVAEKVHNVFQHAEEKAQKPVVPVKDTKAESSTAAGMMIGLSIKR  495

T1P11      PSKLTASPGPIPESLKYLAPNGKYTDWIVNVRAQKHGLGQSFRIIVFLGEFNPDP  550
WHITEHEAD  PSKLTASPGPIPESLKYLAPDGKYTDWIVNVRAQKHGLGQSFRVIVFLGEFNPDP  550
OR         PSKLTASPGPIPESLKYLAPDGKYTDWIVNVRAQKHGLGQSFRVIVFLGEFNPDP  550
TS         PSKLTASPGPIPESLKYLAPDGKYTDWIVNVRAQKHGLGQSFRVIVFLGEFNPDP  550

             ★                                         ↓
T1P11      ET.NDEFNCVGRVSVLGRSAETQCSKCRKDNANGLIVSSTMPLTSALLQDIVGGE  605
WHITEHEAD  ETWDDEFNCVGRVSVLGRSAETQCGKCRKDNANGLIVSGTVPLTSALLQDIVGGE  605
OR         ETWDDEFNCVGRVSVLGRSAETQCGKCRKDNANGLIVSGTVPLTSLCCRILWAAS  605
TS         ETWDDEFNCVGRVSVLGRSAETQCGKCRKDNANGLIVSGTVPLTSLCCRILWAAS  605
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T1P11      LQSLKPKDVIPHLRANLKWKVTLFNGDEYNLKEVPDLKVSVASTEVTIDKEGLPH  660
WHITEHEAD  LQSLKPEDVIPHLRANLKWKVALFNGDEYNLEEVPDLKVSVASTEVTIDEEGLPH  660
OR         SRASSLRMSSRICAPT                                         621
TS         SRASSLRMSSRICAPT                                         621

T1P11      YSRQYTVYPKITEGKPYGHGPEDHI                                685
WHITEHEAD  YSRQYTVYPEITEGKPCGHGPEDHI                                685
```

◆ = *N*-acetylation site

⊓ = Thioether bridge

S = Missense codon

★ = Nonsense codon

⫯ = Cleavage site

↓ = Frameshift generated by polymorphic site (insertion) polyclonal antibody against the cleaved C-terminal portion of the tyrosinase enzyme was able to recognize protyrosinase (see section 1.12; Kupper *et al.*, 1990b). This polyclonal antibody was raised against the C-terminal region from amino acids 412 to 621 of the predicted sequence of strains OR and TS. Of these 209 amino acids, 183 would not be affected by the frameshift-causing insertion, so the novel C-terminus predicted from my sequencing would still be recognized by the antibody. Thus, it seems likely that the base insertion and the resulting alteration in amino acid sequence are actually part of the *bona fide* tyrosinase gene and protein sequence, and that the previous sequencing was in error. Regardless, since the C-terminus of tyrosinase is removed, the insertion does not affect the mature enzyme sequence or activity.

The RIP changes in the *T* gene of strain T1P11 caused 39 missense mutations and 3 nonsense mutations in the predicted amino acid sequence for tyrosinase when compared with the amino acid sequences from the TS and OR strains (Fig. 13). A further 6 missense mutations occurred in the novel 90 amino acid C-terminal region of the predicted protein when compared to the predicted Whitehead sequence. The first nonsense mutation truncated the 407 amino acid mature polypeptide to 72 amino acids and undoubtedly disrupts tyrosinase activity. Furthermore, heavily RIPed genes like the *T* allele in strain T1P11 are often not transcribed (Rountree and Selker, 1997), so the strain most likely carries a functionally null allele of *T*. Subsequent comparison of strain T1P11 with T1P11P08, T1P11P13 and wild-type strain NCN10 during a tyrosinase plate assay revealed that only colonies from T1P11 did not turn brown after several days.

3.7 Construction of second reporter strain

Strain T1P11, confirmed to contain RIP mutations in the *T* gene, was used to develop a second reporter strain by transformation with the reporter construct pBAT. Bleomycin resistant transformants were subjected to the tyrosinase plate assay. After five hours, colonies from several strains had turned brown when grown in the presence of chloramphenicol and had remained white on non-inducing media (Table 7). When the time of incubation in the tyrosine solution was extended to 24 hours, colonies from some of these strains turned light brown on non-inducing media, while colonies from other strains and the parent strain T1P11 remained white. The strains were analyzed by

Table 7.

Tyrosinase assay of potential reporter strains from the transformation of T1P11 with pBAT.

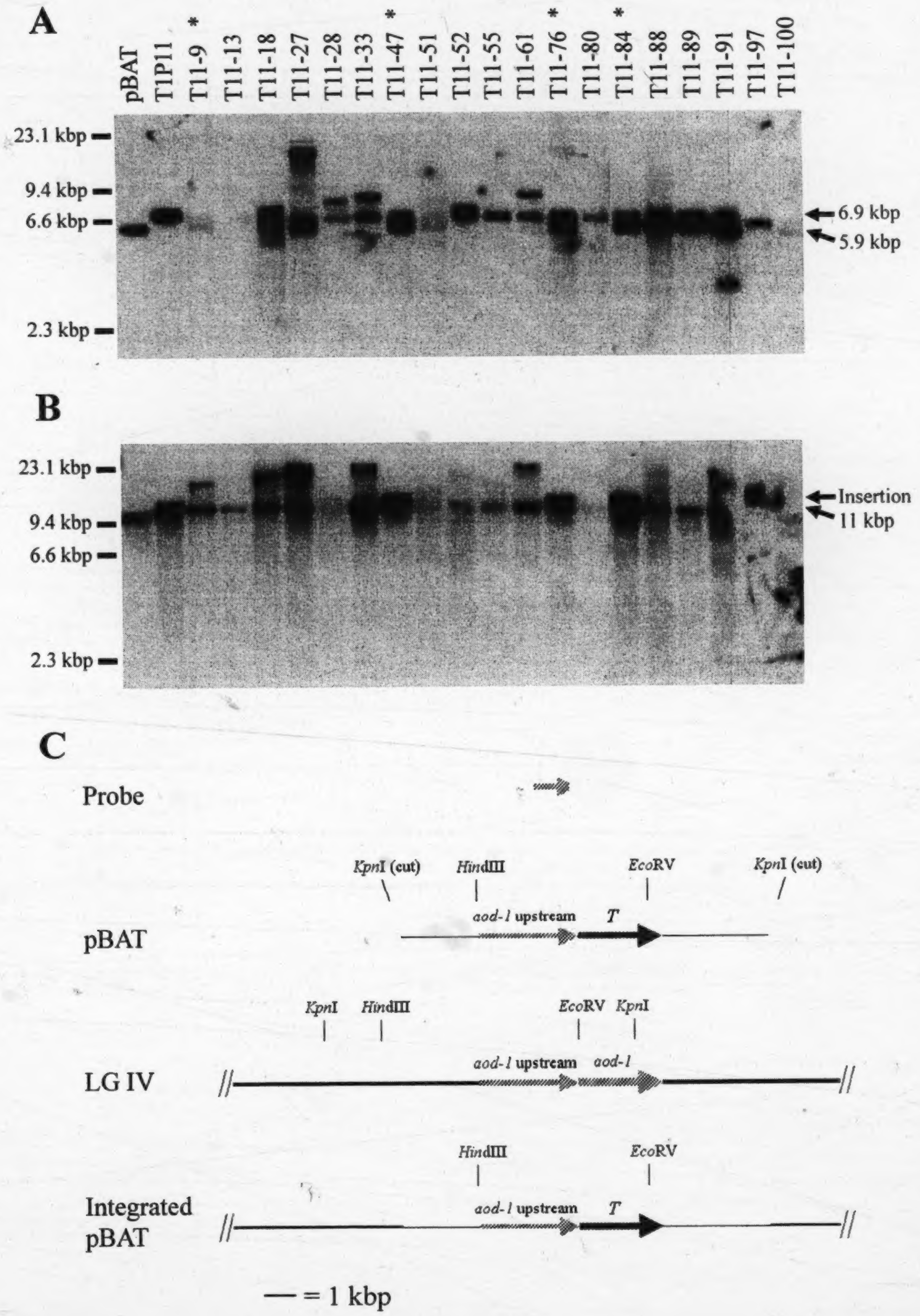
Strain	Five Hours		Twenty-four Hours	
	+ Chloramphenicol	Non-induced	+ Chloramphenicol	Non-induced
T11-9	Brown	White	Dark Brown	Light Brown
T11-13	Brown	White	Dark Brown	Light Brown
T11-18	Brown	White	Dark Brown	Light Brown
T11-27	Brown	White	Dark Brown	Light Brown
T11-28	Brown	White	Dark Brown	Light Brown
T11-33	Brown	White	Dark Brown	White
T11-47	Brown	White	Dark Brown	White
T11-51	Brown	White	Dark Brown	Light Brown
T11-52	Light Brown	White	Dark Brown	Light Brown
T11-55	Brown	White	Dark Brown	Light Brown
T11-61	Light Brown	White	Dark Brown	White
T11-76	Brown	White	Dark Brown	White
T11-80	Brown	White	Dark Brown	White
T11-84	Brown	White	Dark Brown	Light Brown
T11-88	Light Brown	White	Dark Brown	Light Brown
T11-89	Light Brown	White	Dark Brown	White
T11-91	Brown	White	Dark Brown	White
T11-97	Light Brown	White	Dark Brown	Light Brown
T11-100	Brown	White	Dark Brown	Dark Brown

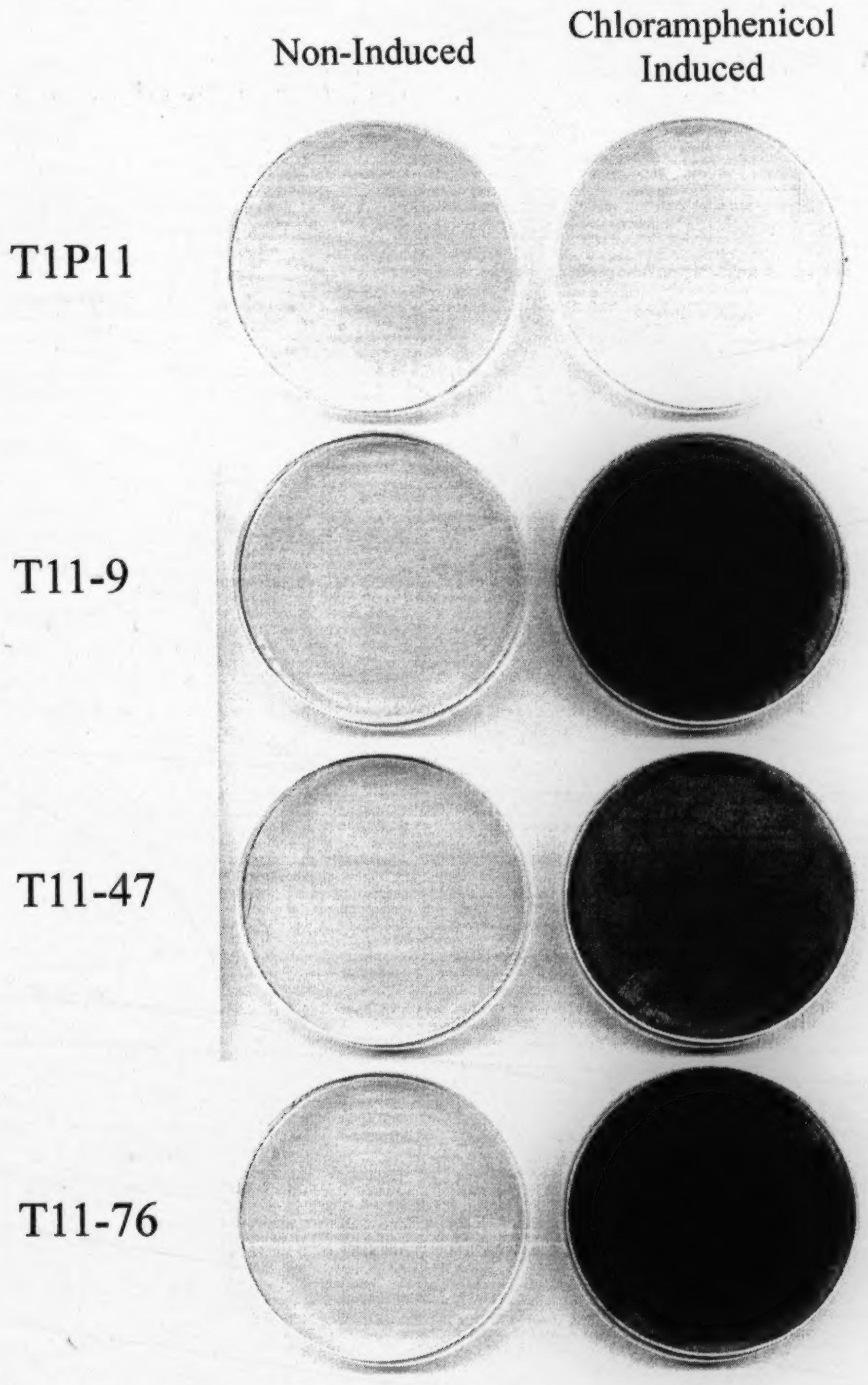
Southern blotting and transformants T11-9, -47, -76, and -84 were found to contain a single inserted copy of the entire reporter (Fig. 14). The tyrosinase plate assay was repeated and, due to the relatively strong and fast colour change exhibited by colonies on inducing media, strain T11-76 was chosen as the reporter strain (Fig. 15). Colonies of this strain grown in the presence of chloramphenicol began to turn brown about one and a half hours after the addition of tyrosine.

3.8 Mutagenesis of strain T11-76

Although the original strategy was to use random insertion of a resistance conferring plasmid to mutagenize the reporter strain, I found that this approach was labour intensive and I was concerned that the integration sites may not be entirely random. Instead, I decided to use EMS to mutagenize the reporter strain. In theory, mutant strains that fail to turn brown in inducing media and those that turn brown under non-inducing conditions could both yield useful alternative oxidase regulatory mutations. However, I decided to limit the screen to mutant strains that did not produce the brown pigment when grown in the presence of chloramphenicol. Mutant strains constitutively expressing tyrosinase could have *aod-1* regulatory mutations or they could have mutations affecting a large number of genes affecting oxidative phosphorylation that would also cause constitutive *aod-1* expression. Therefore, I decided to focus on non-inducing mutants to increase the likelihood of specifically obtaining alternative oxidase regulatory mutations.

Two approaches were used to obtain colonies unable to properly induce tyrosinase expression (Fig. 6). In the first (direct selection), EMS mutagenized conidia were immediately spread on chloramphenicol containing plates. In the second (antimycin A enrichment), mutagenized conidia were subjected to filtration enrichment in liquid medium containing antimycin A. Any conidia able to germinate and grow in the presence of antimycin A should be those still able to induce alternative oxidase and respire via the alternative pathway. Therefore, these germinating conidia were removed daily by filtration until no further growth was observed. The remaining conidia were plated on chloramphenicol containing plates. In both methods, colonies that formed on the chloramphenicol plates were screened for reporter expression by the tyrosinase plate


A
pBAT
T1P11
T11-9 *
T11-13
T11-18
T11-27
T11-28
T11-33
T11-47 *
T11-51
T11-52
T11-55
T11-61
T11-76 *
T11-80
T11-84 *
T11-88
T11-89
T11-91
T11-97
T11-100
23.1 kbp
9.4 kbp
6.6 kbp
2.3 kbp
6.9 kbp
5.9 kbp
B
23.1 kbp
9.4 kbp
6.6 kbp
2.3 kbp
Insertion
11 kbp
C
Probe
KpnI (cut)
HindIII
EcoRV
KpnI (cut)
pBAT
aod-1 upstream
T
KpnI
HindIII
EcoRV
KpnI
LG IV
aod-1 upstream
aod-1
HindIII
EcoRV
Integrated pBAT
aod-1 upstream
T
— = 1 kbp


Non-Induced
Chloramphenicol Induced
T1P11
T11-9
T11-47
T11-76

assay, and white colonies from a background of brown colonies were selected.

Mutagenesis using each approach was performed several times (Table 8). For the purposes of discussing the data, the experimental results from each approach were pooled. In total, about 128, 500 colonies were screened via the first approach, and 1583 colonies were picked that remained white under inducing conditions. After these isolates had formed conidia, they were streaked onto chloramphenicol containing plates and re-screened with the tyrosinase plate assay. 146 of these strains still remained white during the tyrosinase plate assay, as shown for one mutant strain in Fig. 16. Using the second method, about 2 x 10^9 conidia were inoculated into the antimycin A liquid media, and about 7500 colonies grew when plated following the enrichment procedure. These strains were then screened by the plate assay. 116 colonies that remained white were picked, allowed to conidiate in slants, and streaked on chloramphenicol containing plates. Re-screening revealed that 42 of these strains remained white when incubated with the tyrosine solution.

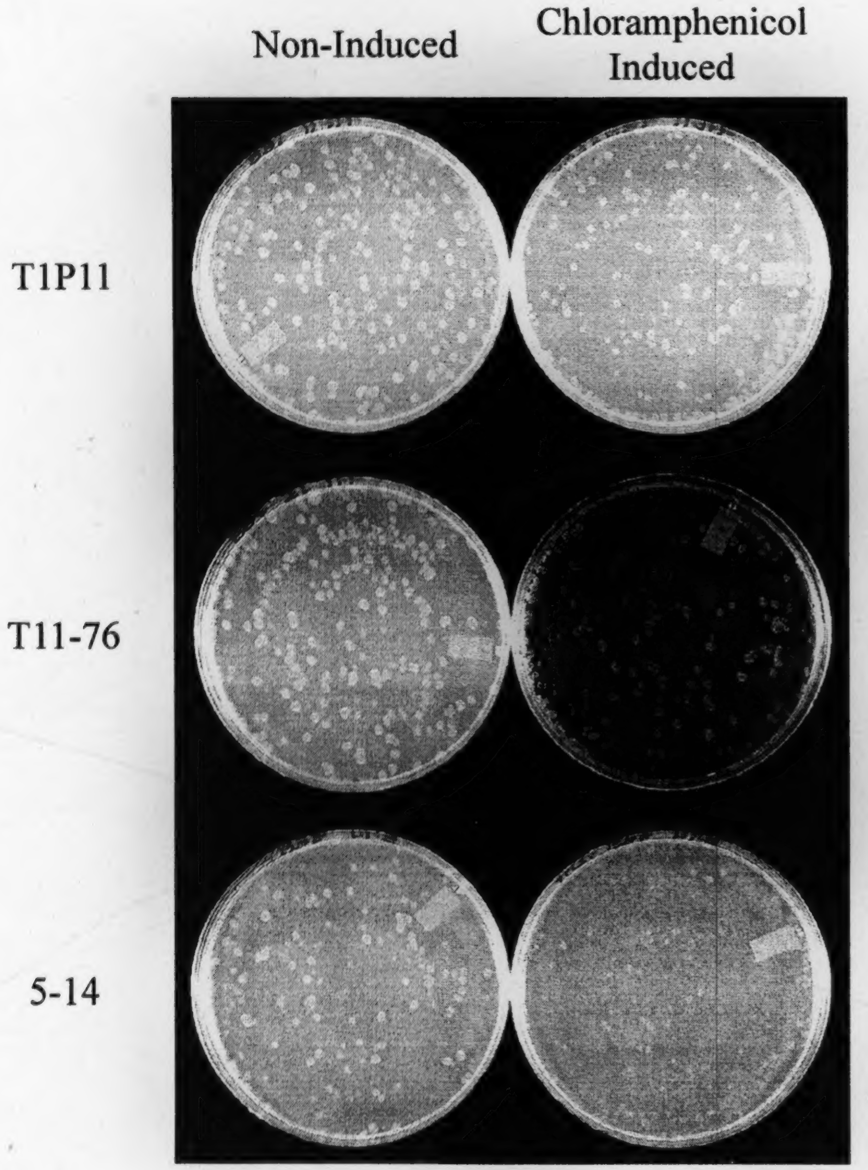
It was possible that strains unable to induce the reporter correctly could contain mutations in the reporter construct itself. To rule out this possibility, I examined the mutants for their ability to induce alternative oxidase activity by assaying for cyanide-insensitive respiration following growth in chloramphenicol. Eighteen mutant strains (carrying mutations designated *E1* through *E18*) unable to induce both the tyrosinase reporter and alternative oxidase activities were selected for further study as putative alternative oxidase regulatory mutations. Six of these mutations (*E1*, *E2*, *E3*, *E4*, *E7*, and *E15*) arose from the direct plating method, while twelve (*E5*, *E6*, *E8*, *E9*, *E10*, *E11*, *E12*, *E13*, *E14*, *E16*, *E17*, and *E18*) arose from the filtration enrichment technique. Finally, it was possible that the mutants did not induce alternative oxidase because they had become resistant to chloramphenicol. To address this issue, cultures of each mutant strain were grown in antimycin A and assayed for the presence of cyanide-insensitive respiration under alternative oxidase inducing conditions. With the exception of strain 4-294 (*E2* mutation), all the mutant strains were also unable to induce alternative oxidase when grown in the presence of antimycin A (and many were, in fact, unable to grow). Thus, seventeen strains carrying mutations affecting alternative oxidase regulation were isolated.

Table 8.
Mutants isolated from EMS mutagenesis.

EMS Method	[EMS] M	Number Colonies Screened[1]	Number Picked	Number Characterized by Respiration	Mutant Strains (mutation name)
1	1	19600	104	18	
1	1	39300	211	16	2-195 (*E1*)
1	1.5	37300	353	40	4-294 (*E2*)
1	3	4400	48	9	5-14 (*E3*), 5-34 (*E4*)
1	3	21900	432	27	6-280 (*E7*)
1	3	5100	402	29	7-64 (*E15*)
1	3	900	33	7	
2	1.5	2×10^8, 600[2]	20	5	L1-6 (*E5*), L1-13 (*E6*)
2	1.5	8×10^8, 4900	71	24	L2-25 (*E8*), L2-37 (*E9*), L2-40 (*E10*), L2-61 (*E11*), L2-62 (*E12*), L2-64 (*E13*), L2-67 (*E14*)
2	1.5	8×10^8, 2000	25	13	L3-4 (*E16*), L3-5 (*E17*), L3-8 (*E18*)
Total Method 1		128 500	1583	146	6
Total Method 2		1.8×10^9, 7500	116	42	12

[1] The number of colonies per plate was counted for 25 plates and the average was used to extrapolate the total colonies screened.

[2] The first number indicates the total number of conidia treated with EMS and inoculated into antimycin A containing liquid medium, while the second number indicates the number of colonies that grew when the remaining conidia were plated after enrichment.

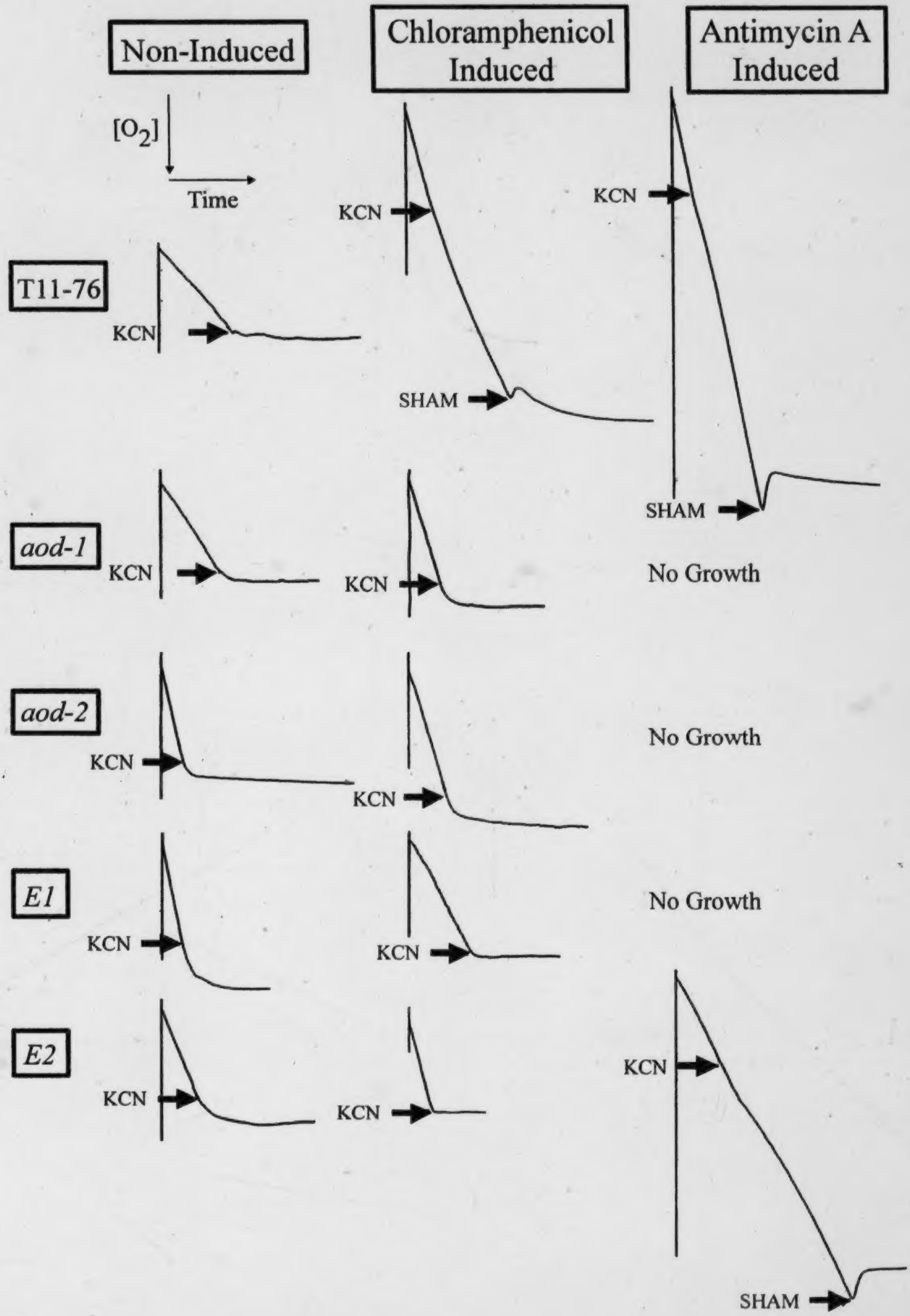

Non-Induced
Chloramphenicol Induced
T1P11
T11-76
5-14

To further characterize the isolated mutations, the eighteen isolated strains were divided between myself and another graduate student in the lab. I focused on strains carrying the mutations *E1*, *E2*, *E3*, *E8*, *E13*, and *E14*. I also did preliminary work with the strain carrying mutation *E12*. The respiratory tracings for these strains grown under non-inducing conditions and grown in the presence of chloramphenicol or antimycin A revealed that mutations *E1*, *E3*, *E8*, *E12*, *E13*, and *E14* prevented alternative oxidase induction by chloramphenicol, while the *E2* mutation prevented alternative oxidase induction by chloramphenicol but not by antimycin A (Fig. 17). Tracings for the previously described *aod-1* and *aod-2* mutant strains (Bertrand *et al.*, 1983) and the reporter strain T11-76 are also shown as controls. Strains carrying the mutations *E1*, *E3*, *E8*, *E12*, *E13*, and *E14*, as well as the *aod-1* and *aod-2* mutant strains, were unable to grow in the presence of antimycin A because this inhibitor blocked respiration through the cytochrome pathway and the mutants were unable to respire through the alternative pathway.

3.8.1 Comparison of methods of EMS mutagenesis

Using the estimate that 70% survival was achieved on average for the EMS mutagenesis (see section 2.28; Table 6), then the 128, 500 surviving colonies screened in the direct plating method arose from about 183, 600 viable conidia originally mutagenized (Table 9). In the antimycin A enrichment method, 1.8 x 10^9 conidia were mutagenized and taken through filter enrichment. With the first method, 6 of 183, 600 or about 3 x 10^{-3} % of the initially treated conidia were isolated as *aod-1* regulatory mutants, while with the second method, only 12 of 1.8 x 10^9 or about 7 x 10^{-7} % of the initially treated conidia were isolated as mutants. This comparison suggests that the first method is approximately 5000 times more effective than the second at generating mutants with a given initial amount of conidia treated.

However, the second method involves much less manipulation, with twice the number of mutants isolated from only 7% of the total number of white colonies picked as compared to the first method. With the first method, about 8% of the initially treated conidia were picked as white colonies, while with the second method, we picked only about 6 x 10^{-6} % of the initially treated conidia. The first method led to identification of


Non-Induced
Chloramphenicol Induced
Antimycin A Induced
[O2]
Time
T11-76
aod-1
aod-2
E1
E2
KCN
SHAM
No Growth
No Growth
No Growth

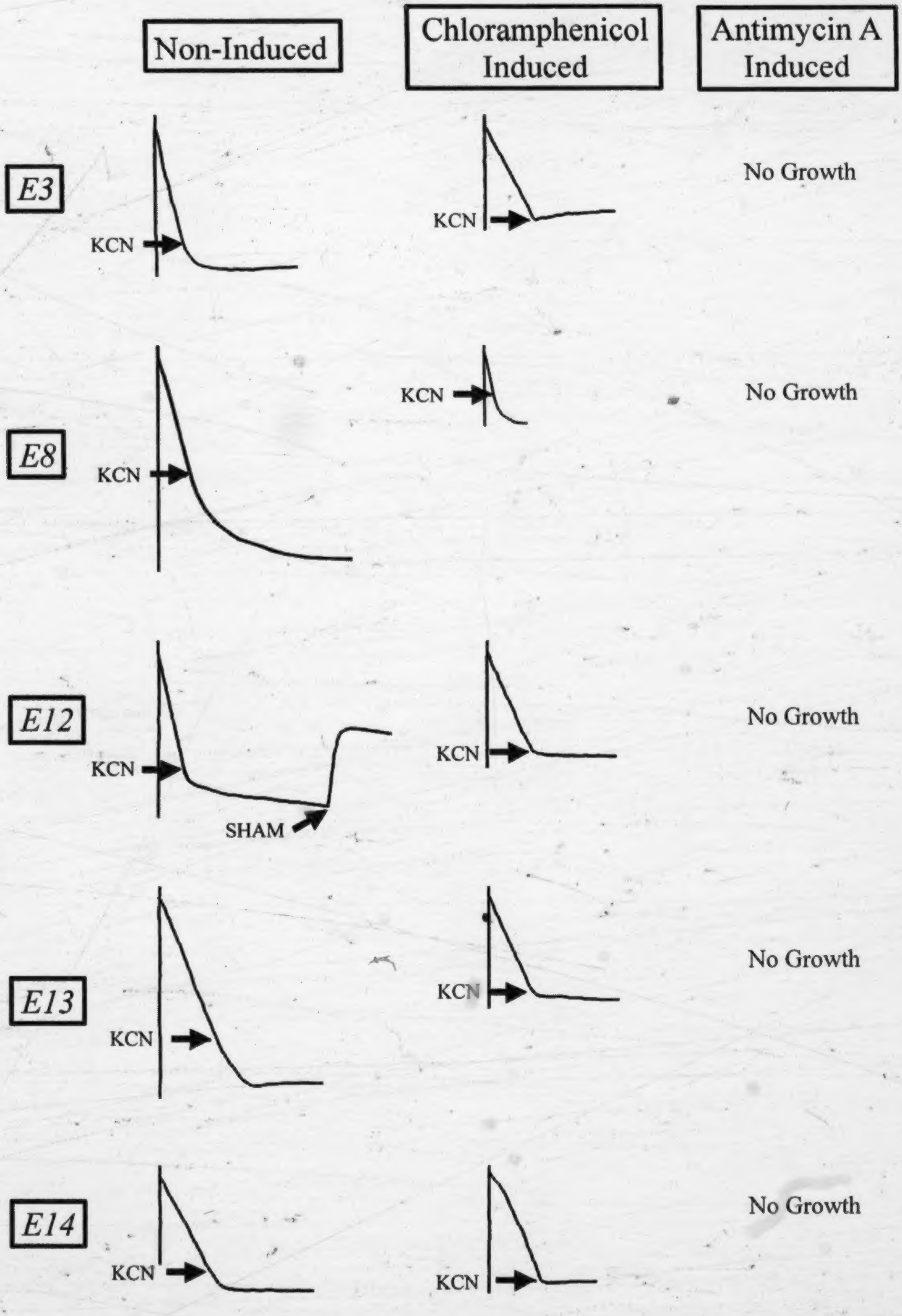

Non-Induced
Chloramphenicol Induced
Antimycin A Induced
E3
KCN
KCN
No Growth
E8
KCN
KCN
No Growth
E12
KCN
SHAM
KCN
No Growth
E13
KCN
KCN
No Growth
E14
KCN
KCN
No Growth

Table 9.

Comparison of EMS mutagenesis methods.

EMS Method	Total Viable Conidia Treated	Viable Conidia Following EMS	White Colonies Picked	White when Retested	Mutants Affecting Endogenous *aod-1* Induction
1	183 600 [1]	128 500	1583	146	6
% of Total		70.0	8.6	8.0 X 10^{-2}	3.3 X 10^{-3}
% of Viable			12.3	1.1 X 10^{-1}	4.7 X 10^{-3}
% of Picked				9.2	3.8 X 10^{-1}
% of Retested					4.1
2	1.8 X 10^{9}	1.26 X 10^{9} [2]	116	42	12
% of Total		70.0	6.4 X 10^{-6}	2.3 X 10^{-6}	6.7 X 10^{-7}
% of Viable			9.2 X 10^{-6}	3.3 X 10^{-6}	9.5 X 10^{-7}
% of Picked				36.2	10.3
% of Retested					28.6

[1] The number of conidia treated was estimated based on the number of viable colonies seen and a 70% survival rate.

[2] The number of viable conidia was estimated based on the number of total conidia treated and a 70% survival rate.

only about 0.4% of the originally picked strains as mutants, while the second method led to the identification of about 10% of the originally picked strains as mutants. Therefore, the second method is more user friendly and efficient than the first, with an approximate 27 fold increase in the likelihood that a white colony picked during the first tyrosinase plate assay will be an *aod-1* regulatory mutant.

3.9 Crossing of mutant strains to obtain progeny

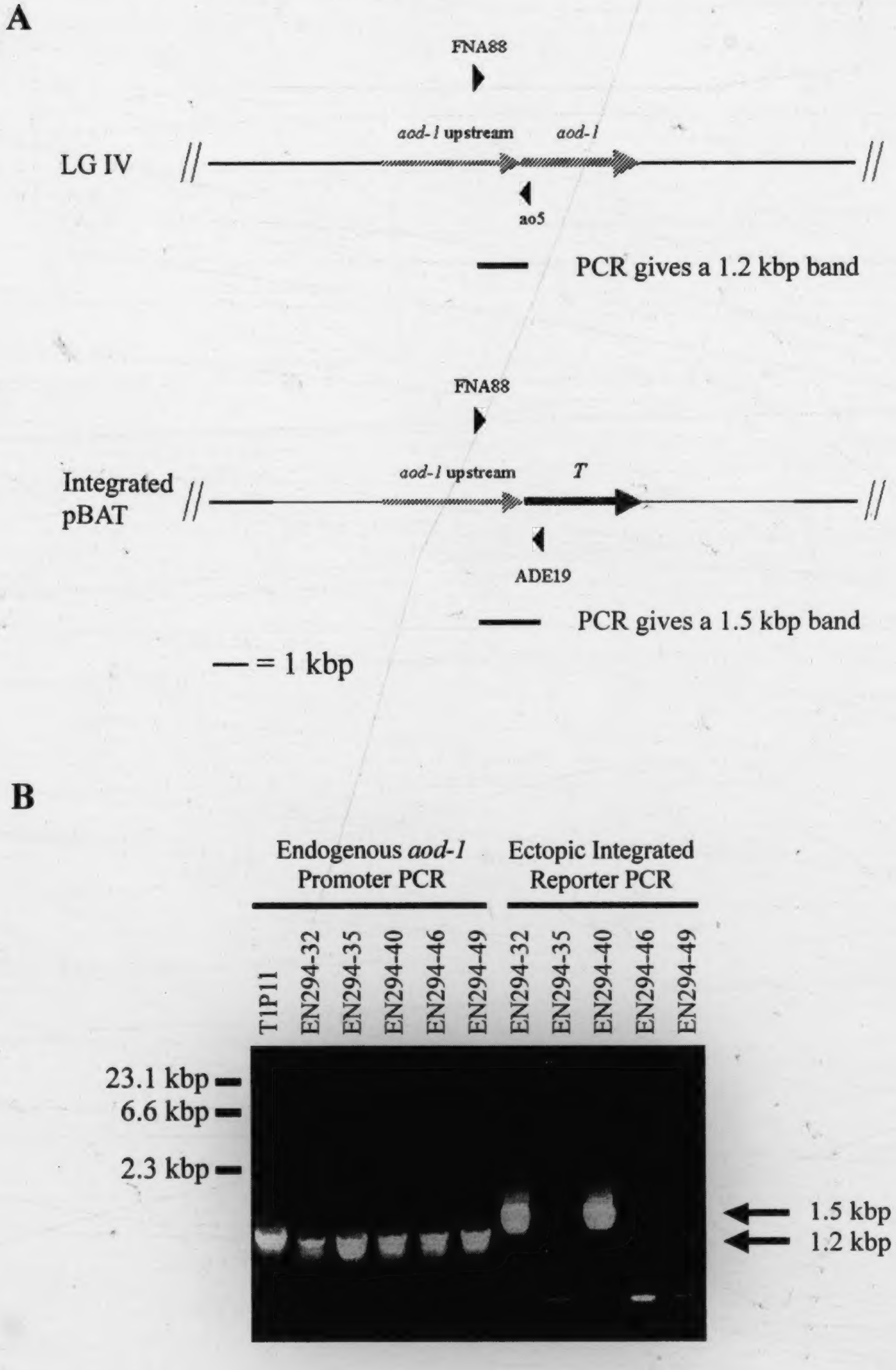
The isolated strains were crossed as males to help remove any secondary EMS mutations that may have occurred. Crosses were also performed with auxotrophic strains to introduce a marker that would allow the formation of forced heterokaryons, for use in complementation tests between different strains, by inoculation of both strains onto minimal media (Table 10; see Table 2 for complete list of progeny). Progeny were screened and isolates identified that contained both the mutation of interest and the auxotrophic marker. The isolated mutations were all inherited in a Mendelian fashion, indicating that they are in nuclear encoded genes.

Since the original mutant isolates used in these crosses contained the integrated reporter construct, it was possible that the endogenous and ectopic *aod-1* upstream sequences could be substrates for RIP, which affects duplicated sequences in the *N. crassa* genome during a sexual cross (Selker, 1990). Thus, progeny from crosses involving a strain still carrying the reporter construct could contain RIP mutations in the endogenous *aod-1* promoter, which could lead to lack of expression of the structural gene. Therefore, the endogenous *aod-1* upstream region in the progeny being considered for further work was amplified by PCR (Fig. 18), and the product was sequenced. Only those isolates with a wild-type endogenous *aod-1* upstream sequence were used for further analysis. This insured that a lack of induction of alternative oxidase activity in progeny from crosses was due to the regulatory mutation of interest and not inactivation of expression of the *aod-1* locus itself. Typically, of fifty progeny examined, five to ten carried the requirement and the mutation of interest, and were sequenced to insure the endogenous alternative oxidase promoter was intact. Roughly 75% of the strains sequenced contained a non-RIPed *aod-1* upstream sequence (data not shown).

Table 10.
Crosses of mutant strains to introduce auxotrophic markers.

Auxotrophic Strain (♀)	Mutant Strain (♂)	Progeny	Desired Genotype
NCN233	2-195 (*E1*)	EN195-__ [1]	*E1, pan-2*
NCN246	4-294 (*E2*)	EN294-__	*E2, pyr-6*
7263	5-14 (*E3*)	EN14-__	*E3, ad-2*
7267	L2-25 (*E8*)	EL25-__	*E8, met-7*
7262	L2-62 (*E12*)	EL62-__	*E12, trp-3*
7266	L2-64 (*E13*)	EL64-__	*E13, ad-1*
7261	L2-67 (*E14*)	EL67-__	*E14, ad-3A*

[1] Progeny isolated from each cross were numbered (represented by underline) and individual progeny used for further work are listed in Table 2.


A
FNA88
aod-1 upstream
aod-1
LG IV
ao5
PCR gives a 1.2 kbp band
FNA88
aod-1 upstream
T
Integrated pBAT
ADE19
PCR gives a 1.5 kbp band
— = 1 kbp
B
Endogenous aod-1 Promoter PCR
Ectopic Integrated Reporter PCR
T1P11
EN294-32
EN294-35
EN294-40
EN294-46
EN294-49
EN294-32
EN294-35
EN294-40
EN294-46
EN294-49
23.1 kbp
6.6 kbp
2.3 kbp
1.5 kbp
1.2 kbp

To prevent the need for repeated sequencing of the *aod-1* upstream region in progeny of subsequent crosses, isolates were chosen that had lost the ectopically integrated reporter construct through random segregation. The presence or absence of the reporter construct was determined by examination of genomic DNA for the presence of the *aod-1/T* fusion by PCR, using a primer for the *T* gene and a primer for the *aod-1* upstream region (Fig. 18). Another concern was that the reporter strain T11-76 and all of the original mutant isolates derived from this strain contained the *al-2* mutation. Since plans for genetic mapping of the mutations involved crosses to other strains that carry the albino locus *al-1* as a marker for linkage group I, non-albino progeny were selected from the original set of crosses. Finally, the growth rates of different progeny from a single cross were variable, possibly due to the presence in the original EMS isolate of other mutations affecting growth that had randomly assorted to the offspring. Whenever possible, progeny were selected with the most rapid growth rate, closest to the reporter strain T11-76.

3.10 Complementation analysis

Complementation analysis was done to determine if the regulatory mutations carried by the strains obtained were allelic to each other or to the previously described regulatory gene *aod-2*. If the regulatory mutations in a heterokaryon are allelic then they will fail to complement, resulting in a lack of cyanide-insensitive respiration even under alternative oxidase inducing conditions. The analysis was performed by creating heterokaryotic strains, in which the two types of nuclei present each contained one of the isolated mutations and a different auxotrophic marker. The differing auxotrophic markers insured formation and maintenance of heterokaryons on minimal medium.

Complementation analysis showed that we had isolated four novel complementation groups involved in regulating alternative oxidase: *E1* and its alleles, *E10*, *E12*, and *E15*. The complementation is summarized in Table 11, followed by the raw data shown in Fig. 19. These complementation groups have been assigned the gene names *aod-4*, *aod-5*, *aod-6*, and *aod-7*, respectively (Table 12). The *aod-4* gene was the most frequently mutated, with isolates from both method 1 (*E1*) and method 2 (*E8*, *E9*, *E11*, *E13*, and *E14*). Each of the other complementation groups had only a single mutant

Table 11.

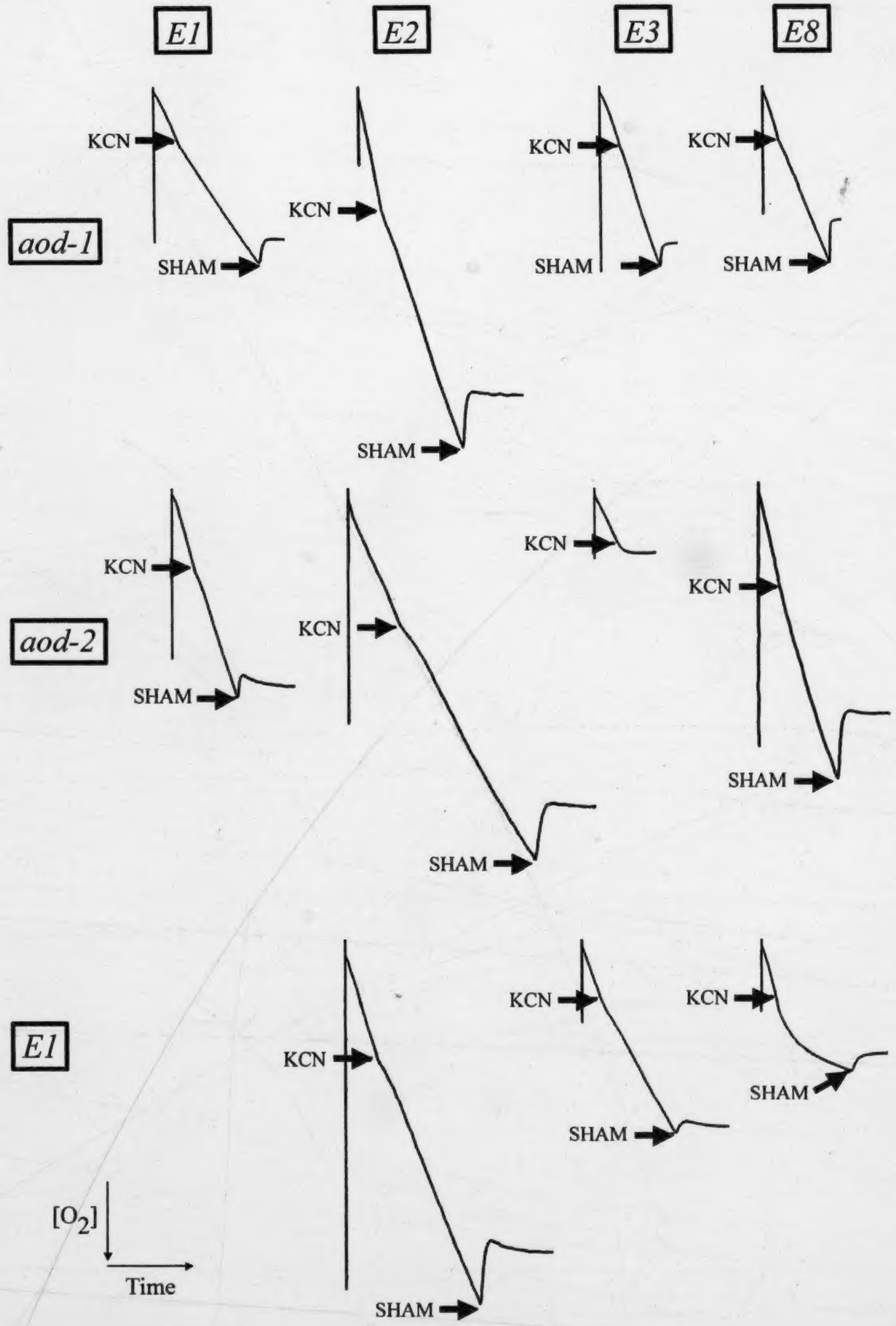
Complementation analysis using heterokaryotic strains.

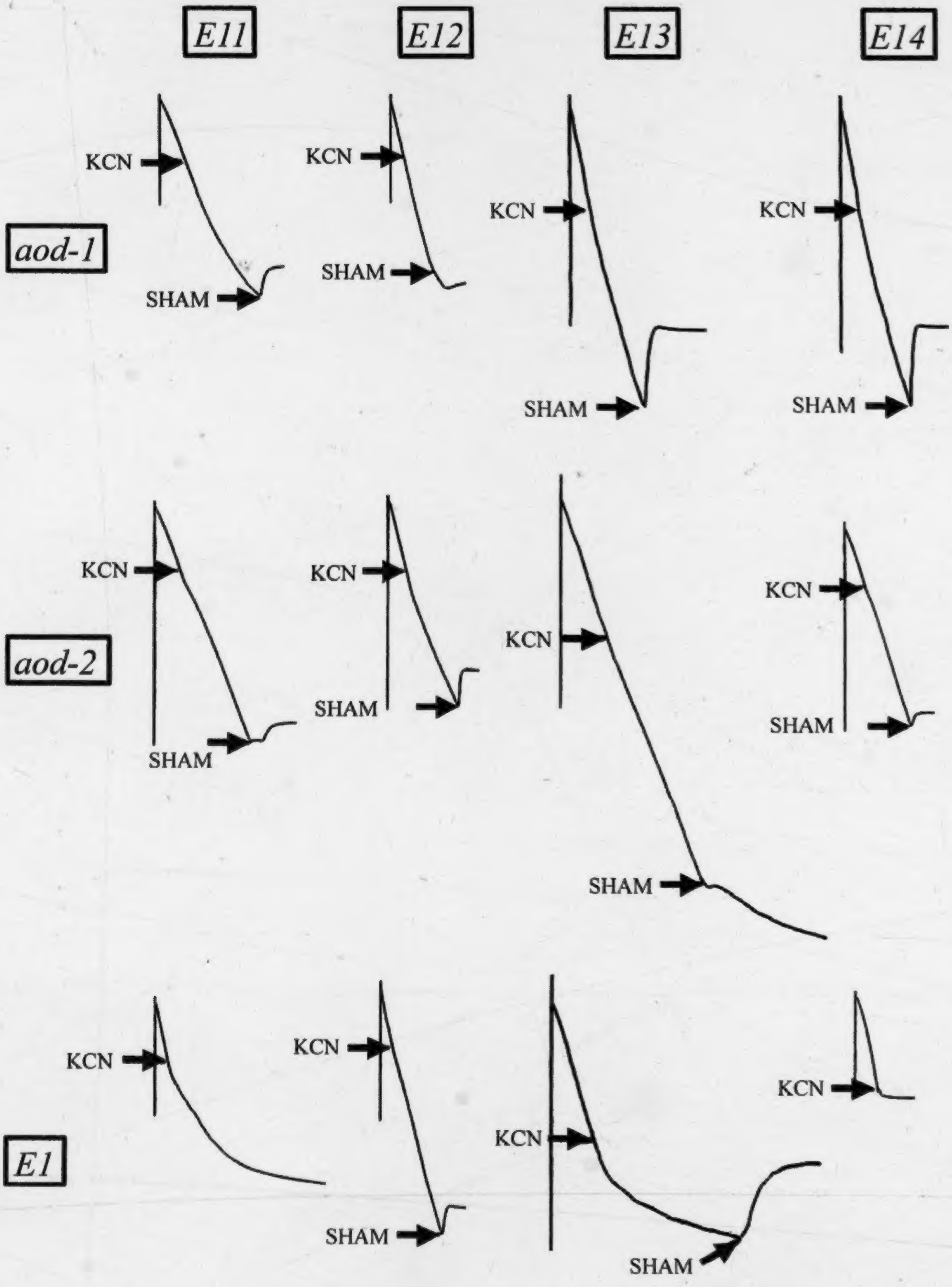
Mutant Strain	**Mutant Strains**										
	E1	***E2***	***E3***	***E8***	***E9*** [2]	***E10***	***E11***	***E12***	***E13***	***E14***	***E15***
aod-1	✓ [1]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
aod-2	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓
E1		✓	✓	×	×	ND[3]	×	✓	×	×	✓
E2			✓	✓	✓	✓	✓	✓	✓	✓	✓
E3				✓	✓	ND	✓	✓	✓	✓	✓
E8					×	ND	×	✓	×	×	✓
E9						✓	×	✓	×	×	✓
E10							✓	✓	ND	ND	✓
E11								✓	×	×	✓
E12									✓	✓	✓
E13										×	✓
E14											ND

[1] Heterokaryons were made between the strains listed for the appropriate row and column. Complementation is indicated by a check mark, non-complementation by an X.

[2] The lightly shaded areas were assayed by another graduate student in the lab (I. Cleary).

[3] ND indicates no data, since once it was discovered that mutations *E1*, *E8*, *E9*, *E11*, *E13*, and *E14* were allelic, and mutations *aod-2* and *E3* were allelic, not all combinations with each of these mutations were analyzed (I. Cleary).


E1
E2
E3
E8
aod-1
aod-2
E1
KCN
SHAM
[O$_2$]
Time


E11
E12
E13
E14
aod-1
KCN
SHAM
KCN
SHAM
KCN
SHAM
KCN
SHAM
aod-2
KCN
SHAM
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SHAM
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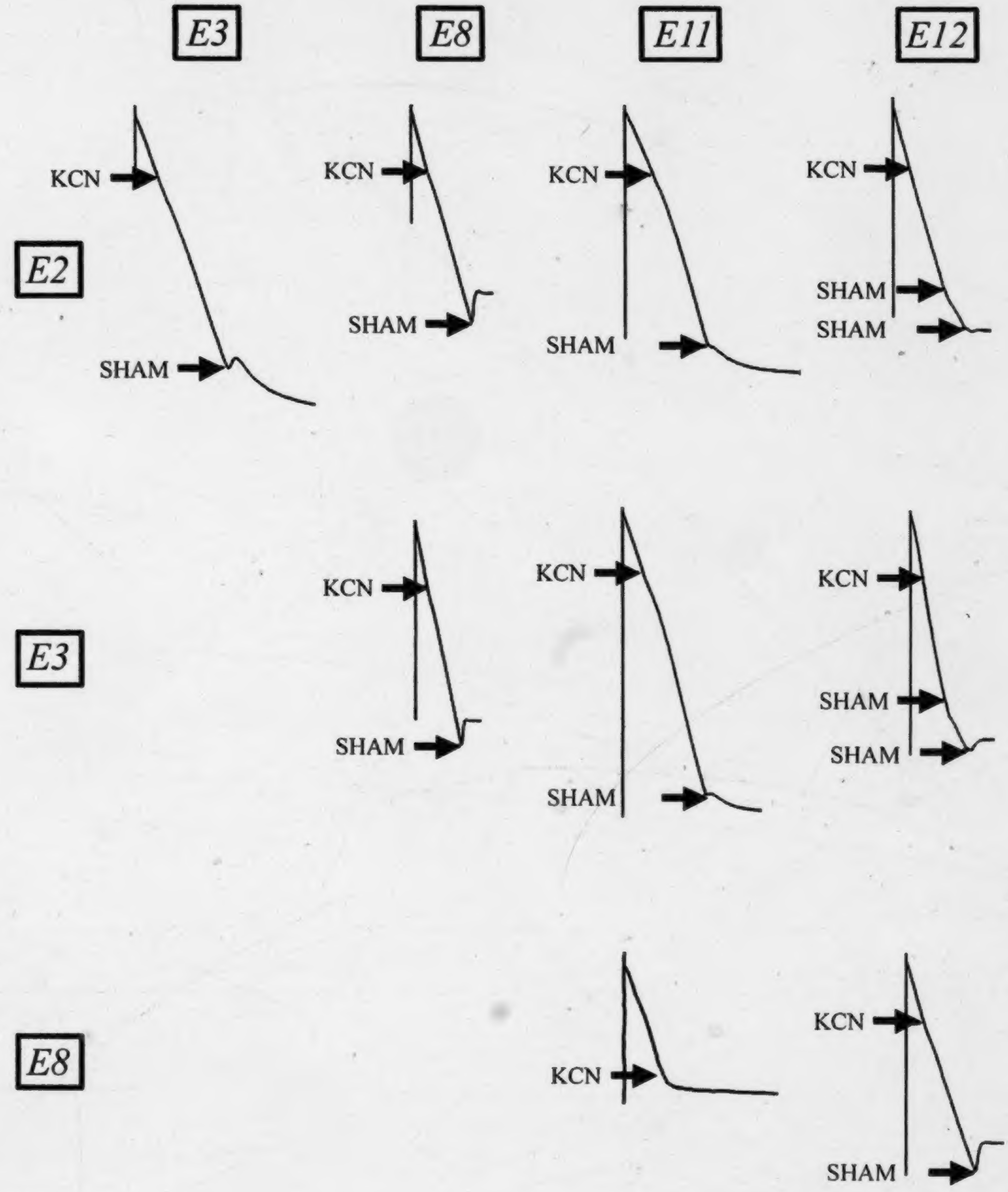
SHAM
KCN
SHAM
E1
KCN
KCN
SHAM
KCN
SHAM
KCN


E3
E8
E11
E12
E2
KCN
SHAM
KCN
SHAM
KCN
SHAM
KCN
SHAM
SHAM
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SHAM
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SHAM
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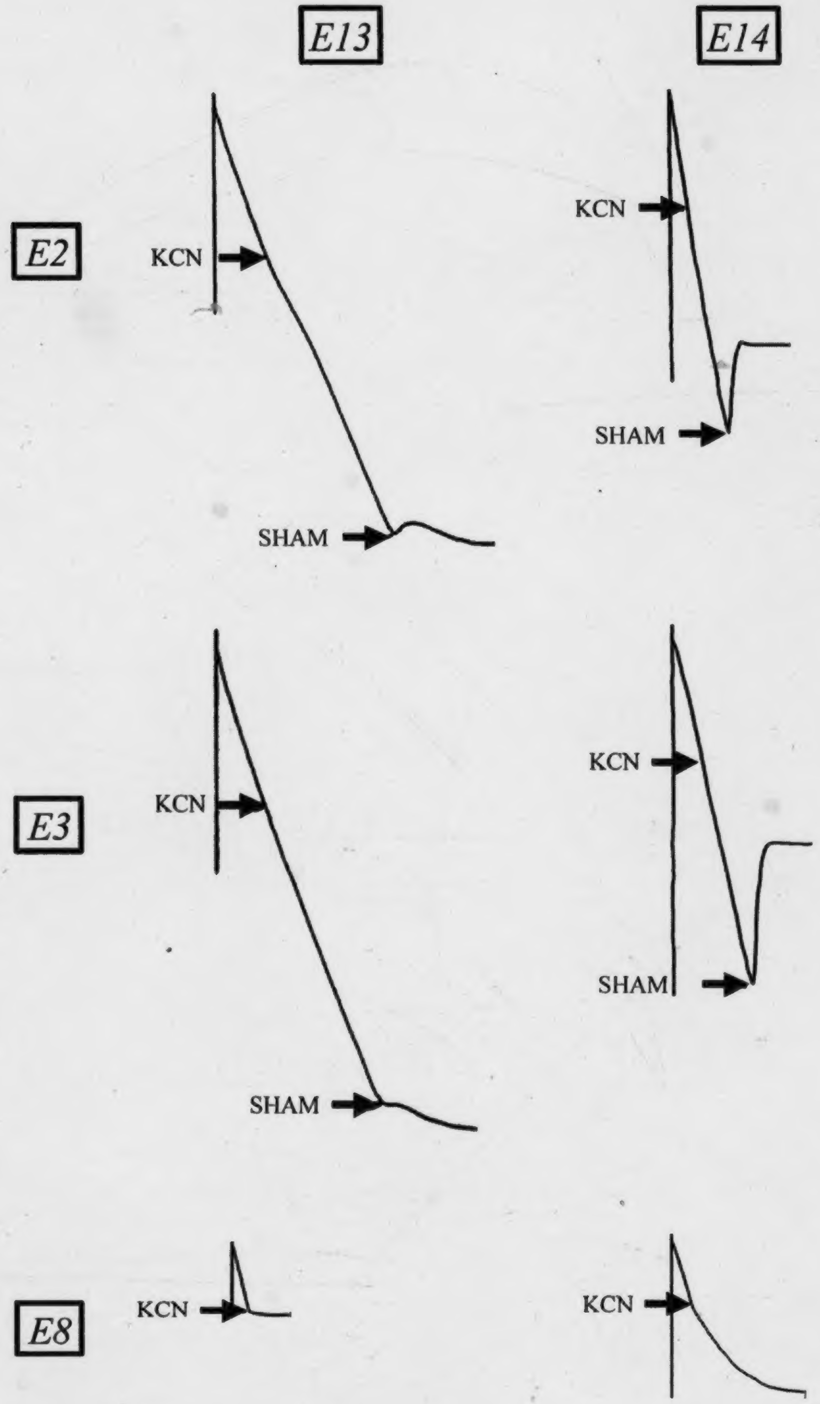
SHAM
SHAM
E8
KCN
KCN
SHAM


E13
E14
E2
KCN
SHAM
KCN
SHAM
E3
KCN
SHAM
KCN
SHAM
E8
KCN
KCN

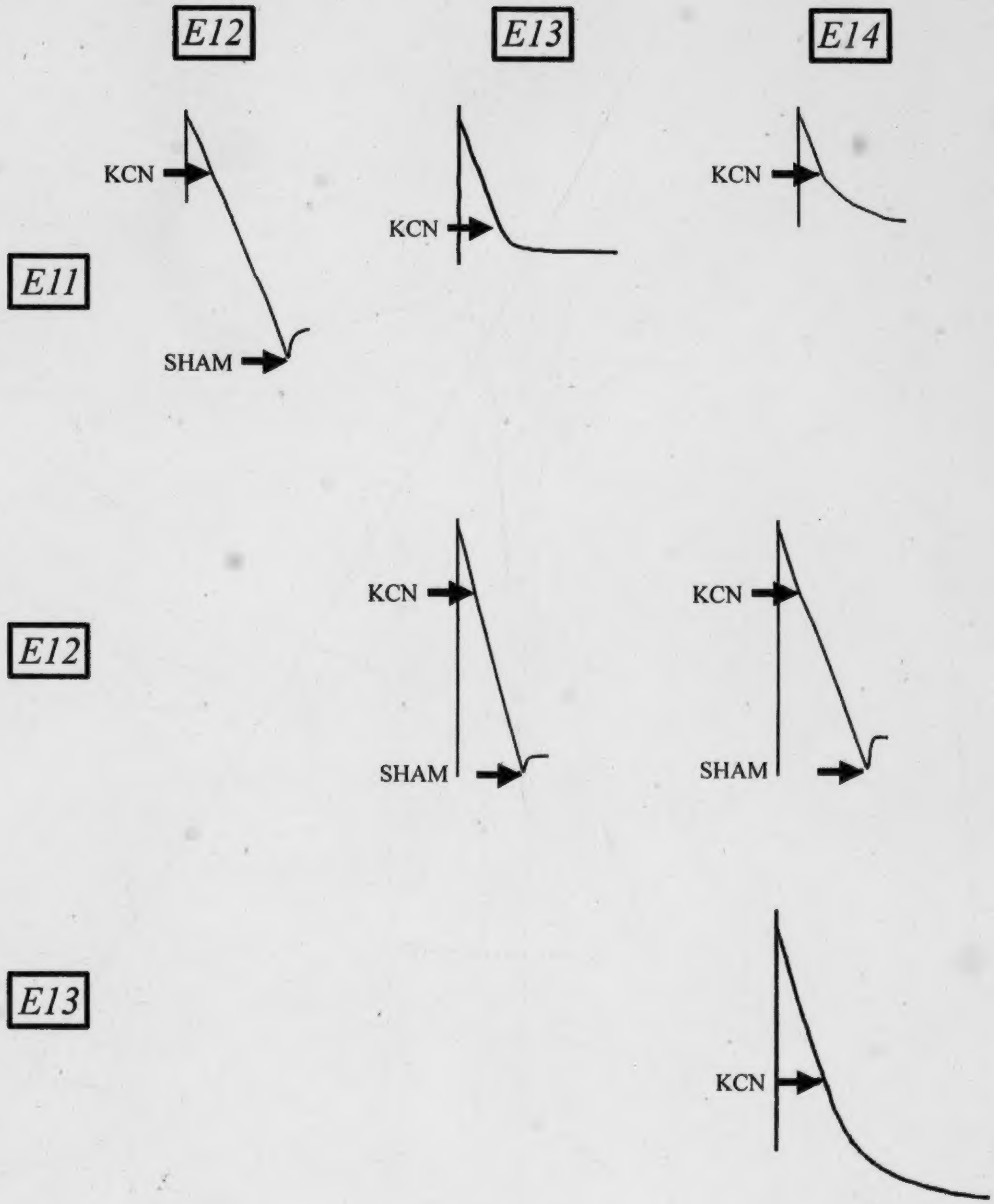

E12
E13
E14
KCN
SHAM
KCN
KCN
E11
KCN
KCN
E12
SHAM
SHAM
E13
KCN

Table 12.
Assignment of *aod* gene designations to new alternative oxidase regulatory mutant complementation groups.

Mutations in Complementation Group	***aod* Gene Designation**	**Strains Used for Further Characterization**
E1, *E8*, *E9*, *E11*, *E13*, *E14*	*aod-4*	EN195-109, 2E1a-57, NL61-130, E11a-40
E10[1]	*aod-5*	
E12	*aod-6*	EL62-2, EL62-25
E15[1]	*aod-7*	

[1] Mutations *E10* and *E15* are being further characterized by another graduate student in the lab (I. Cleary).

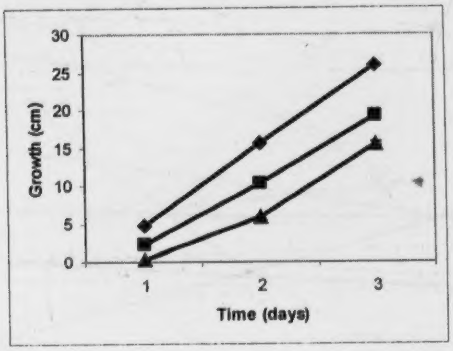
allele. The *E2* mutation represents a fifth complementation group, called *chl-1*, and appears to be a chloramphenicol resistant mutation rather than an alternative oxidase regulatory mutation (see sections 3.8, 3.11 and 3.12). The *E3* mutant strain failed to complement the previously identified regulatory mutant, *aod-2* (Bertrand *et al.*, 1983), and so is likely a new allele of this gene. All of the mutant strains analyzed complemented *aod-1*, confirming that in each strain the endogenous *aod-1* gene was functional. Each mutation analyzed also complemented at least one other mutation, suggesting that none of the isolated mutations was dominant over a wild-type allele of the affected gene. Seven mutations (*E4*, *E5*, *E6*, *E7*, *E16*, *E17*, and *E18*) remain to be characterized by complementation analysis.

Two of the heterokaryotic strains gave respirometer tracings that appear to have slight alternative oxidase activity, as they show a low level of continued respiration after the addition of cyanide (Fig. 19, heterokaryons *E1* + *E8* and *E1* + *E13*). However, this small amount of cyanide-insensitive respiration is likely not due to complementation since homokaryotic strains carrying mutations in the *aod-4* complementation group occasionally give respirometer tracings showing a similar low level of cyanide-insensitive respiration. In fact, control *aod-4* strains examined on the same day as these two heterokaryons showed a similar level of cyanide-insensitive respiration (data not shown). Taken together, these data show that *E1* and *E8*, and *E1* and *E13* do not complement and are alleles of the same gene.

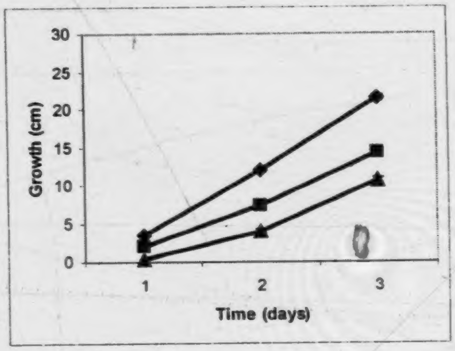
3.11 Growth rates

The growth rates of the *aod-4* and *chl-1* mutant strains under alternative oxidase inducing or non-inducing conditions, at both 30°C and room temperature, were determined and compared to the alternative oxidase wild-type strain NCN246 and the reporter strain T11-76 (Fig. 20, 21). As expected, the growth rate of NCN246 was reduced by growth in the presence of chloramphenicol or antimycin A, with antimycin A having a stronger effect. A decreased incubation temperature also caused the growth rate of NCN246 to decrease. Strain T11-76 grew slightly slower than NCN246 under all conditions. The *aod-1* and *aod-2* strains had growth rates similar to NCN246 and T11-76 at both incubation temperatures when grown on non-inducing or chloramphenicol

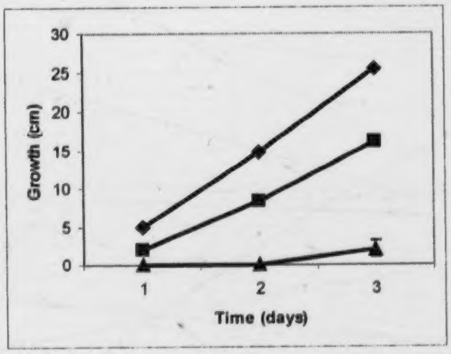
NCN246



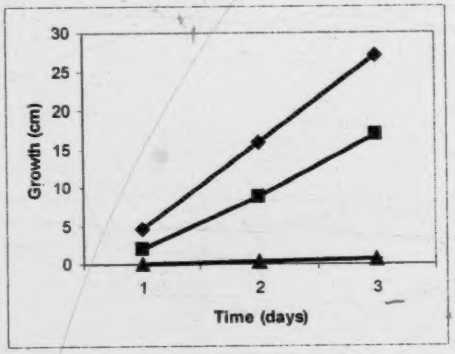
T11-76



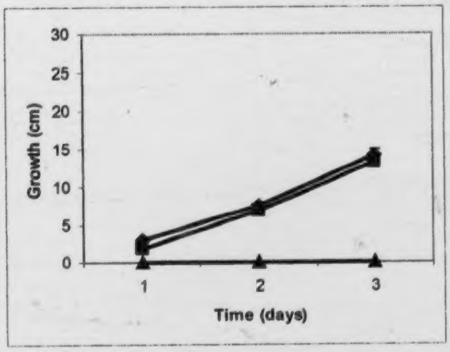
aod-1



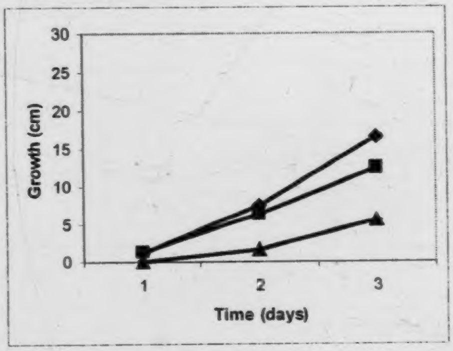
aod-2



aod-4 (EN195-109)

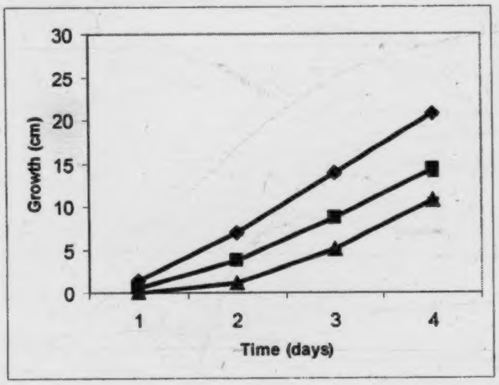


chl-1 (E2a-119)

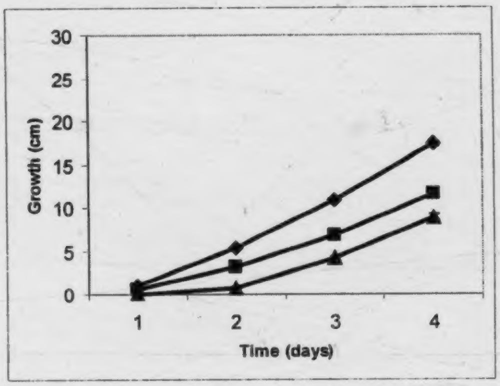


◆ = Non-inducing ■ = Chloramphenicol ▲ = Antimycin A

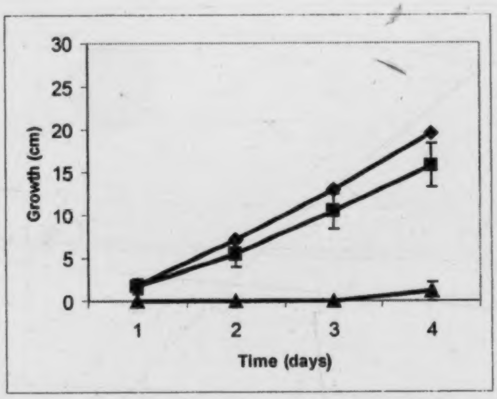
NCN246



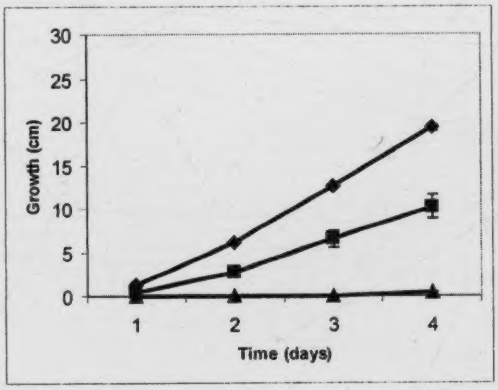
T11-76



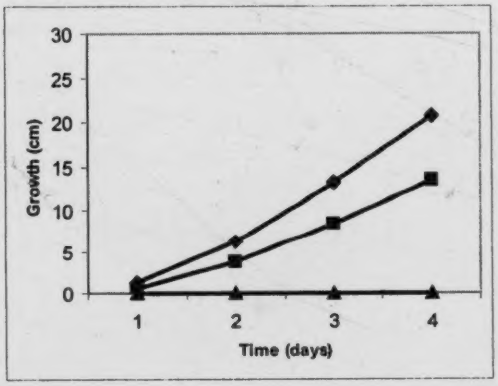
aod-1



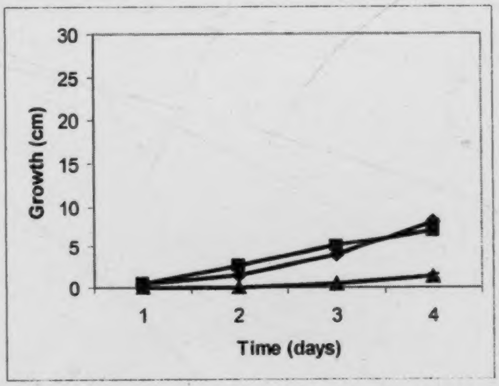
aod-2



aod-4 (EN195-109)



chl-1 (E2a-119)



◆ = Non-inducing ■ = Chloramphenicol ▲ = Antimycin A containing media. However, the *aod-1* and *aod-2* strains showed little to no growth in antimycin A containing media, which is expected for strains lacking alternative oxidase activity because antimycin A completely inhibits electron transfer through complex III. On the other hand, chloramphenicol does not fully block the synthesis of mitochondrial translation products. Therefore, in the presence of chloramphenicol some respiration can occur through the cytochrome pathway.

The *aod-4* strain EN195-109 had a diminished growth rate on non-inducing media at 30°C, reducing the growth rate to that observed for the same strain in the presence of chloramphenicol. It is unclear why chloramphenicol did not further reduce the growth rate for this strain at 30°C. Perhaps the use of race tubes overlooked subtle differences in growth rates under these two conditions, which might be revealed by growth in liquid culture. This growth defect was not seen when the strain was grown at room temperature. At this temperature, the presence of chloramphenicol also caused the expected decrease in growth rate in the *aod-4* strain, similar to that seen with NCN246, T11-76, *aod-1*, and *aod-2* strains. Like the *aod-1* and *aod-2* strains, the *aod-4* strain did not grow in the presence of antimycin A.

Compared to T11-76, the *chl-1* strain E2a-119 had a slightly reduced growth rate at 30°C on all tested media. This reduction in growth rate was exacerbated by incubation at room temperature. During the first two days of growth at 30°C and over the four days of growth at room temperature, the growth rates on non-inducing and chloramphenicol containing medium were virtually identical. This similarity is most likely because the mutation in *chl-1* confers resistance to chloramphenicol. Continued measurement of the growth rate at room temperature revealed that in the presence of chloramphenicol, the growth rates of *chl-1* strains EN294-46 and E2a-119 showed a distinct reduction after about 7 days in the race tubes. As expected from our respiration studies, the *chl-1* strains were the only isolated mutant strains that were able to grow on antimycin A. On this inhibitor, the initial growth rate was reduced as compared to growth on non-inducing media, to an extent similar to that of wild-type strains.

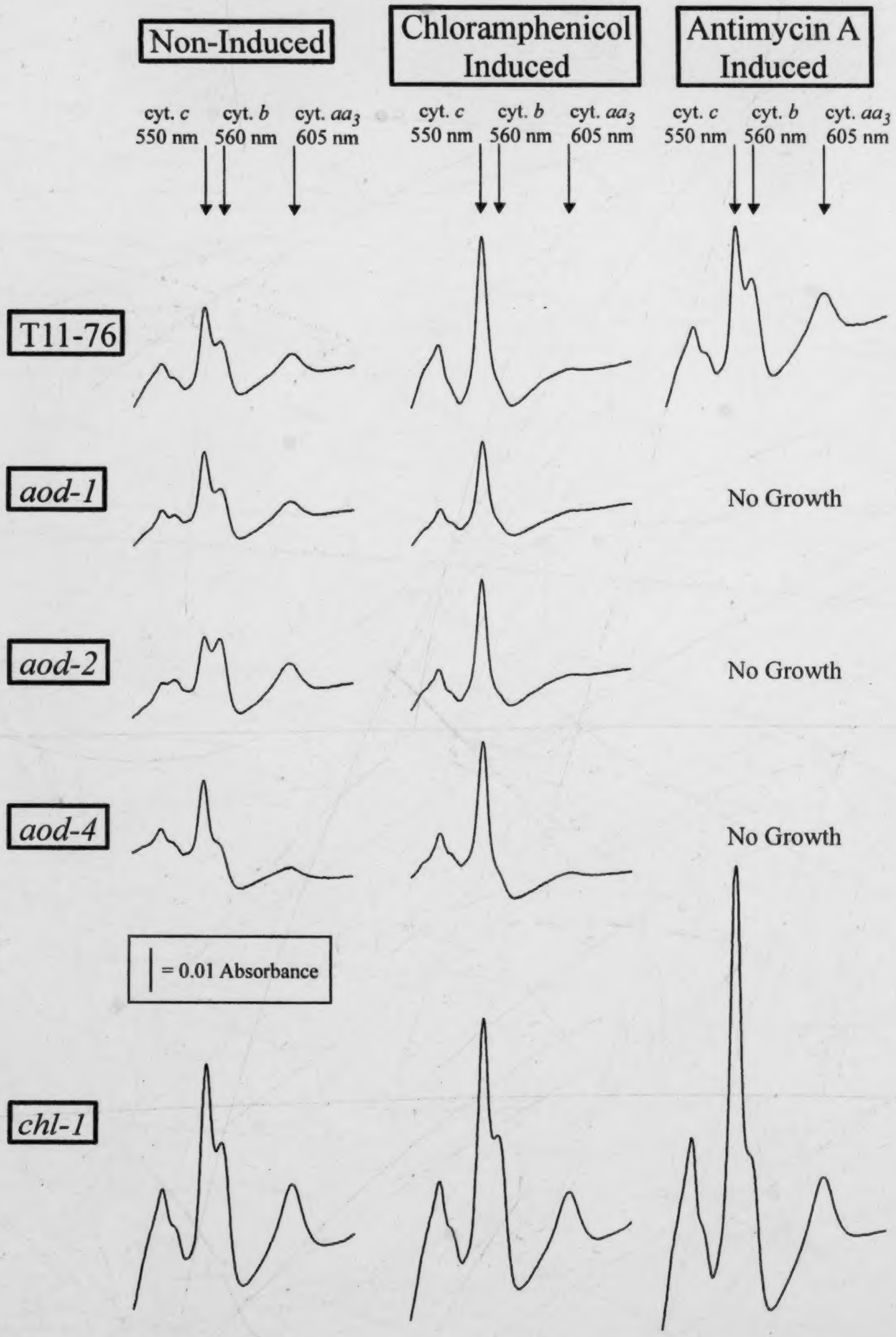
3.12 Cytochrome spectra

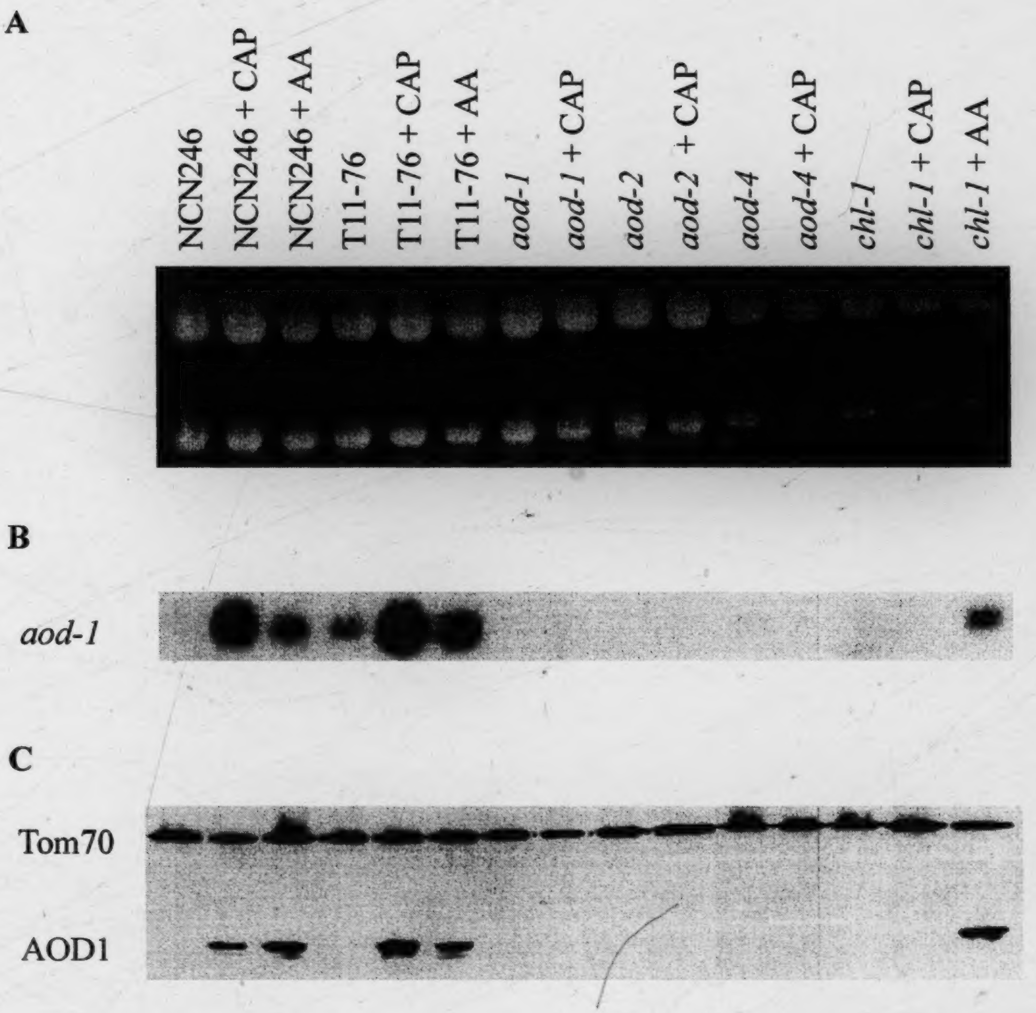
Cytochrome spectra were obtained for the *aod-4* and *chl-1* mutant strains to determine if the alternative oxidase regulatory mutations had any affect on these components of electron transport (Fig. 22). When grown in non-inducing conditions, the *aod-4* strain had a partial deficiency of cytochromes aa_3 and *b*. This reduction suggests that the *aod-4* mutation also affects other aspects of mitochondrial function, and may explain why the *aod-4* strain exhibited a reduced growth rate on non-inducing media at 30°C. As expected, the levels of cytochromes aa_3 and *b* were reduced even further when the strain was grown in the presence of chloramphenicol, since both of these cytochromes contain components synthesized on mitochondrial ribosomes (see section 3.1). Thus, the aberrant growth rate for this strain when grown under non-inducing conditions at 30°C, as compared to growth in the presence of chloramphenicol at 30°C, cannot be completely explained by the levels of cytochromes aa_3 and *b*, since chloramphenicol did cause a further reduction in these cytochromes but not in the growth rate.

The reporter strain T11-76, as well as the *aod-1* and *aod-2* strains, also showed the expected reductions in cytochromes aa_3 and *b* when grown in the presence of chloramphenicol. However, the levels of these cytochromes in strain *chl-1* did not change appreciably when chloramphenicol was present, in agreement with the observation that this strain exhibited only cyanide-sensitive respiration when grown in the presence of chloramphenicol, and consistent with the notion that the mutation confers chloramphenicol resistance. Unlike the *aod-1*, *aod-2*, or *aod-4* mutant strains, the *chl-1* mutant strain was able to grow in the presence of antimycin A, and its spectrum following growth in this condition was similar to the control strain. All strains exhibited variable increases in cytochrome *c* content following growth in either inhibitor. Increased levels of cytochrome *c* are typical of strains that have reduced electron transfer due to inhibitors or mutations affecting complexes III and/or IV (Bertrand *et al.*, 1977).

3.13 Alternative oxidase expression

The *aod-4* and *chl-1* mutant strains were also examined for expression of *aod-1* mRNA and protein by Northern and Western analysis (Fig. 23). As expected from previous studies, the amount of *aod-1* mRNA present in wild-type cultures increased


Non-Induced
Chloramphenicol Induced
Antimycin A Induced
cyt. *c* 550 nm
cyt. *b* 560 nm
cyt. aa_3 605 nm
T11-76
aod-1
aod-2
aod-4
chl-1
No Growth
No Growth
No Growth
= 0.01 Absorbance


A
NCN246
NCN246 + CAP
NCN246 + AA
T11-76
T11-76 + CAP
T11-76 + AA
aod-1
aod-1 + CAP
aod-2
aod-2 + CAP
aod-4
aod-4 + CAP
chl-1
chl-1 + CAP
chl-1 + AA
B
aod-1
C
Tom70
AOD1

dramatically upon growth in media containing chloramphenicol or antimycin A (Li *et al.*, 1996; Tanton *et al.*, 2003). This increase correlated with the appearance of AOD1 protein (Fig. 23; Li *et al.*, 1996; Tanton *et al.*, 2003). Surprisingly, we found that the tyrosinase mutant strain T1P11 (data not shown) and the reporter strain T11-76 (Fig. 23) differ from other strains that are wild-type with respect to respiration, because they contain significant levels of *aod-1* transcript under non-inducing conditions. However, even though the mRNA is present, there is no AOD1 protein in these non-induced strains and there is no alternative oxidase activity (Fig. 23, data not shown). The *aod-4* strain EN195-109 contains no *aod-1* transcript and no AOD1 protein when grown in either inducing or non-inducing conditions, further confirming that the mutation in this strain affects the regulation of alternative oxidase (Fig. 23). The *chl-1* strain EN294-46 contains both alternative oxidase transcript and the AOD1 protein only when grown in the presence of antimycin A. Long exposure of the Northern blots revealed that these mutant strains contain small amounts of *aod-1* mRNA under all conditions, at levels similar to the *aod-1* and *aod-2* strains (data not shown).

3.14 Mapping of the regulatory mutations

The isolated mutations were mapped to specific regions of the seven *N. crassa* linkage groups so that attempts to clone the genes involved would be simplified. By delineating the probable location of the mutations, cosmids already localized to these regions in established cosmid libraries (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003) could be used for transformation and phenotypic rescue, hopefully facilitating the identification of the mutations. The locations of the regulatory mutations on the genetic map were determined in a series of genetic crosses. First, the strains were crossed with *alcoy* linkage tester strains, which carry a series of translocations coupled to specific markers that allow the assignment of a gene to either linkage groups I or II, III or VI, or IV or V (Perkins *et al.*, 1969). Once the possible linkage groups were known, crosses with linkage specific marker carrying strains were performed. The alternative oxidase structural gene *aod-1* and the regulatory gene *aod-2* were previously mapped near the centromeres of linkage groups IV and II, respectively (Bertrand *et al.*, 1983).

Mutations *E1* and *E11* of the *aod-4* complementation group mapped to the right arm of linkage group V (Table 13; Fig. 24A). The *aod-4* gene is most likely located between the *lys-2* and *his-6* markers, near the *inl* marker. Linkage to *inl* was determined to be between 1 and 35% recombination, depending on the cross. This was a surprisingly large discrepancy, but some mapping distances have been reported to vary about 10 fold in crosses involving the same regions (markers) when using strains with different genetic backgrounds (Davis, 2000). Indeed, strains carrying different alleles of the *rec-1* locus have been shown to affect recombination on the right arm of linkage group V approximately 30 fold (Catcheside and Austin, 1969). Thus, the recombination frequencies of 1 to 35% between the *aod-4* alleles and *inl* were near the previously seen range of variation in recombination frequencies for this region. The nature of the *rec-1* locus in the different strains used in the crosses shown in Table 13 is unknown.

Mutation *E2* of the *chl-1* complementation group was closely linked to *nuc-2*, a marker on the right arm of linkage group II, with only 3% recombination observed between the genes (Table 14; Fig. 24B). The data suggested that *chl-1* was most likely located between the *arg-5* and *arg-12* markers.

Mutation *E3* mapped to linkage group II, with 8% recombination with the marker *arg-5* (Table 15; Fig. 24B). The recombination frequency between *E3* and *arg-5* was similar to the recombination frequency of 7% reported for *aod-2* and *arg-5* (Bertrand *et al.*, 1983), further supporting the identification of *E3* as an allele of *aod-2* as suggested by the lack of complementation in the *E3*, *aod-2* heterokaryon (see section 3.10).

Preliminary mapping of mutation *E12* with the *alcoy* strain did not indicate on which linkage group the *aod-6* gene would be found, since the recombination frequencies suggested that the *E12* mutation was not linked to any of the *alcoy* markers (Table 16). The *aod-6* gene could be located on linkage group VII, which is not marked in the *alcoy* strain, or it could fall on one of the ends of the arms of the other linkage groups, with a large physical distance from any *alcoy* marker and translocation site (Davis, 2000).

3.15 Rescue and identification of isolated mutations

To isolate and define the nature of the genes affected in the alternative oxidase regulatory mutants and the chloramphenicol resistant mutant, I wished to screen

Table 13.
Mapping of the *aod-4* gene.

Mated Strains	Number of Progeny	Marker	Number of Parental	Number of Recombinant	Percentage Recombinant	*p* Value (χ^2 Analysis)
997 (*alcoy*, *A*) x EN195-109 (*aod-4*, *pan-2*, *a*)	50	*al-1*	27	23	46	< 0.75
		cot-1	42	8	16	< 0.001
		ylo-1[1] (27)	13	14	52	< 0.90
998 (*alcoy*, *a*) x NL61-130 (*aod-4*, *nic-1*, *A*)	59	*al-1*	21	38	64	< 0.05
		cot-1	46	13	22	< 0.001
		ylo-1 (24)	50	9	38	< 0.25
EN195-109 (*aod-4*, *pan-2*, *a*) x 1243 (*inl*, *cot-1*, *A*)	140	*inl*	129	11	8	< 0.001
		cot-1	68	72	51	< 0.75
NL61-130 (*aod-4*, *nic-1*, *A*) x 1244 (*inl*, *cot-1*, *a*)	148	*inl*	141	7	5	< 0.001
		cot-1	85	63	43	< 0.10
EN195-109 (*aod-4*, *pan-2*, *a*) x 3752 (*caf-1*, *al-3*, *his-6*, *A*)	100	*caf-1*	70	30	30	< 0.001
		al-3	78	22	22	< 0.001
		his-6	68	32	32	< 0.001

Table 13 continued

Mated Strains	Number of Progeny	Marker	Number of Parental	Number of Recombinant	Percentage Recombinant	p Value (χ^2 Analysis)
NL61-130 (*aod-4*, *nic-1*, *A*) x 3753 (*caf-1*, *al-3*, *his-6*, *a*)	104	*caf-1*	78	26	25	< 0.001
		al-3	96	8	8	< 0.001
		his-6	75	29	28	< 0.001
2E1a-57 (*aod-4*, *inl*, *cot-1*, *A*) x 7094 (*mcb*, *a*)	99	*inl*	76	23	23	< 0.001
		mcb	84	15	15	< 0.001
E11a-40 (*aod-4*, *inl*, *A*) x 7094 (*mcb*, *a*)	95	*inl*	62	33	35	< 0.005
		mcb	56	39	41	< 0.10
2E1a-57 (*aod-4*, *inl*, *cot-1*, *A*) x 3753 (*caf-1*, *al-3*, *his-6*, *a*)	89	*al-3*	85	4	5	< 0.001
		inl	88	1	1	< 0.001
		his-6	74	15	17	< 0.001
2E1a-57 (*aod-4*, *inl*, *cot-1*, *A*) x 7255 (*cyh-2*, *lys-2*, *leu-2*, *mei-2*, *a*)	94	*lys-2*	81	13	14	< 0.001
		leu-2	82	12	13	< 0.001
		inl	92	2	2	< 0.001

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[1] Yellow can only be scored amongst al^+ progeny, the number of which is indicated in brackets.

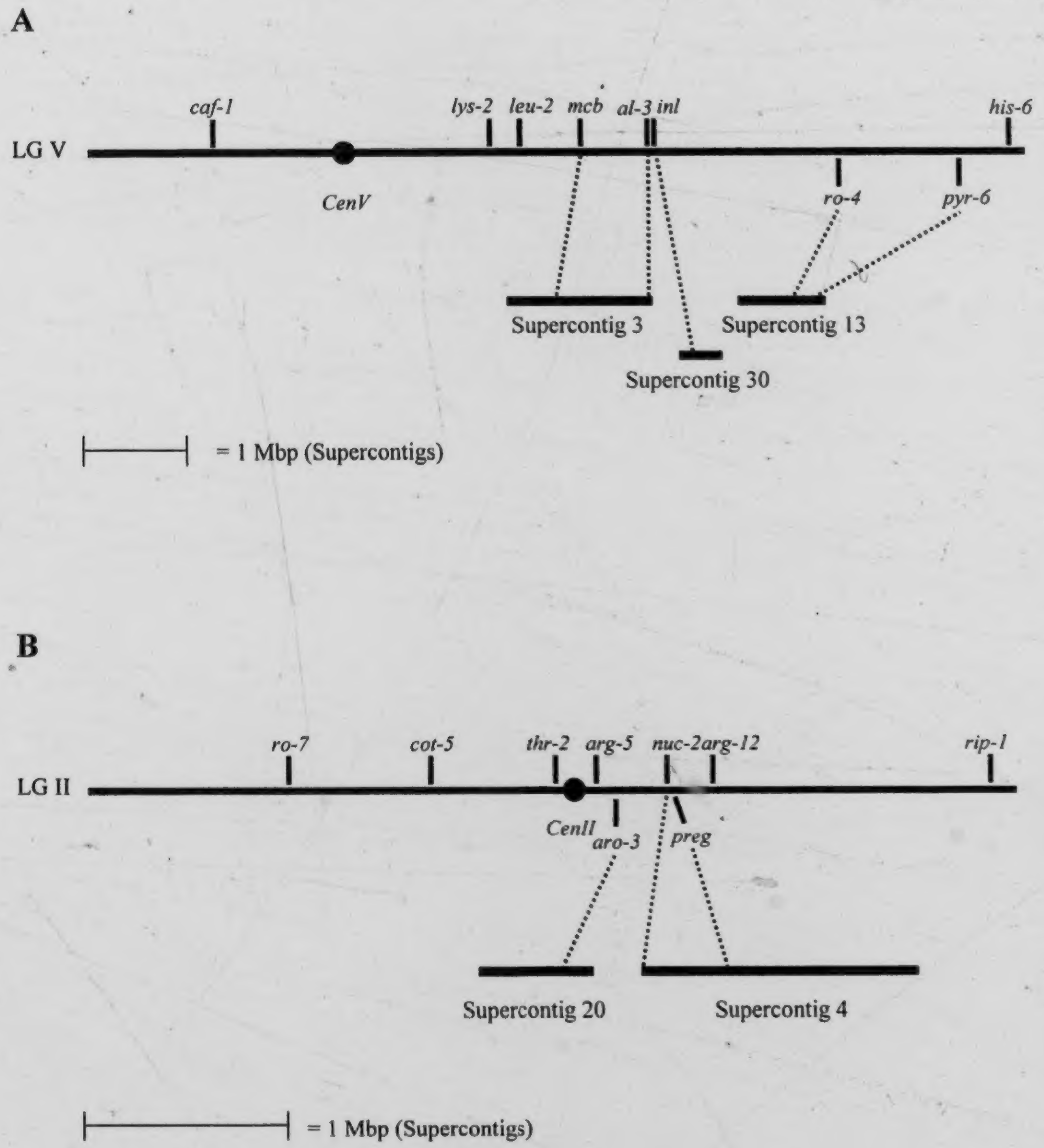
A
caf-1
lys-2
leu-2
mcb
al-3
inl
his-6
LG V
CenV
ro-4
pyr-6
Supercontig 3
Supercontig 13
Supercontig 30
= 1 Mbp (Supercontigs)
B
ro-7
cot-5
thr-2
arg-5
nuc-2
arg-12
rip-1
LG II
CenII
aro-3
preg
Supercontig 20
Supercontig 4
= 1 Mbp (Supercontigs)

Table 14.
Mapping of the *chl-1* gene.

Mated Strains	Number of Progeny	Marker	Number of Parental	Number of Recombinant	Percentage Recombinant	p Value (χ^2 Analysis)
998 (*alcoy*, *a*) x EN294-46 (*chl-1*, *pyr-6*, *A*)	50	*al-1*	50	0	0	< 0.001
		cot-1	24	26	52	< 0.90
		ylo-1[1] (21)	8	13	62	< 0.50
1206 (*al-1*, *arg-5*, *a*) x EN294-46 (*chl-1*, *pyr-6*, *A*)	70	*al-1*	35	35	50	> 0.995
		arg-5	42	28	40	< 0.10
EN294-46 (*chl-1*, *pyr-6*, *A*) x 7356 (*cot-5*, *het-COR*, *pyr-4, thr-2*, *a*)	93	*cot-5*	59	34	37	< 0.10
		thr-2	67	26	28	< 0.001
EN294-46 (*chl-1*, *pyr-6*, *A*) x 6564 (*ro-7*, *arg-5*, *rip-1*, *a*)	46	*ro-7*	30	16	35	< 0.05
		arg-5	36	10	22	< 0.001
		rip-1	23	23	50	> 0.995
E2a-119 (*chl-1*, *arg-5*, *pyr-6*, *A*) x 7356 (*cot-5*, *het-COR*, *pyr-4, thr-2*, *a*)	50	*cot-5*	21	29	58	< 0.25
		thr-2	28	22	44	< 0.50
		arg-5	40	10	20	< 0.001

Table 14 continued

Mated Strains	**Number of Progeny**	**Marker**	**Number of Parental**	**Number of Recombinant**	**Percentage Recombinant**	***p* Value (χ^2 Analysis)**
2998 (*pyr-4*, *arg-12*, *a*) x EN294-46 (*chl-1*, *pyr-6*, *A*)	50	*arg-12*	42	8	16	< 0.001
nuc-2 (*nuc-2*, *a*) x E2a-119 (*chl-1*, *arg-5*, *pyr-6*, *A*)	100	*arg-5*	86	14	14	< 0.001
		nuc-2	97	3	3	< 0.001

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[1] Yellow can only be scored amongst al^+ progeny, the number of which is indicated in brackets.

Table 15.
Mapping of the *E3* mutation.

Mated Strains	Number of Progeny	Marker	Number of Parental	Number of Recombinant	Percentage Recombinant	p Value (χ^2 Analysis)
998 (*alcoy*, *a*) x EN14-158 (*aod-2*, *ad-2*, *A*)	50	*al-1*	42	8	16	< 0.001
		cot-1	24	26	52	< 0.90
		ylo-1[1] (30)	13	17	57	< 0.50
1206 (*al-1*, *arg-5*, *a*) x EN14-158 (*aod-2*, *ad-2*, *A*)	50	*al-1*	29	21	42	< 0.50
		arg-5	46	4	8	< 0.001

[1] Yellow can only be scored amongst al^+ progeny, the number of which is indicated in brackets.

Table 16.
Mapping of the *aod-6* gene.

Mated Strains	Number of Progeny	Marker	Number of Parental	Number of Recombinant	Percentage Recombinant	p Value (χ^2 Analysis)
998 (*alcoy*, *a*) x EL62-2 (*aod-6*, *trp-4*, *A*)	42	*al-1*	26	16	38	< 0.25
		cot-1	17	25	60	< 0.25
		ylo-1[1] (16)	5	9	56	< 0.75

[1] Yellow can only be scored amongst *al*$^+$ progeny, the number of which is indicated in brackets.

established *N. crassa* genomic libraries for clones with the ability to restore inducible alternative respiration to the mutant strains. Since the isolated mutations had been roughly mapped, I was able to focus on specific regions of the genome to locate the appropriate clones. The theory for rescuing a mutant gene is based on the premise that transformation of a mutant strain with a wild-type copy of the altered gene should rescue the ability of that strain either to grow in the presence of antimycin A, in the case of the alternative oxidase regulatory mutations, or to induce alternative respiration in the presence of chloramphenicol, in the case of the *chl-1* mutation. Previous work had shown that transformation of an *aod-1* mutant strain with a wild-type copy of *aod-1* was able to restore chloramphenicol inducible alternative oxidase activity (Tanton *et al.*, 2003), so this approach seemed reasonable.

Genetic mapping placed the *aod-4* gene on the right arm of linkage group V, with 1-35% recombination with the marker *inl* (see section 3.14; Table 13; Fig. 24A). Supercontigs 3, 30 and 13 of the *N. crassa* genome sequence were assembled and placed in this region, and cover about 2.6 Mbp of sequence (Fig. 24A; assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003). It seems likely that the *aod-4* gene is located within this region. Cosmids and bacterial artificial chromosomes (BACs) containing inserts from this region were identified in the genome sequencing project (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003). Most of the 2.6 Mbp region can be covered with three fold redundancy using 214 available cosmids and 32 available BACs from established libraries. However, there are sections totaling approximately 36 kbp that are not covered by any available library, as sequencing was done using a whole genome shotgun approach (Galagan *et al.*, 2003). Furthermore, there are two gaps of unknown size between the three supercontigs, and the *aod-4* gene could be located within one of these gaps. Nevertheless, I decided to attempt to rescue *aod-4* by transforming a mutant strain with clones derived from this region.

Initial attempts at gene rescue were done via electroporation. Tests of transformation frequency using the hygromycin resistance marker showed that the transformation was much more successful if the resistance gene was carried on a linear fragment less than 10 kbp (data not shown). Thus, I analyzed the sequence of 135 cosmids and 21 BACs, which cover approximately 2 Mbp of the region likely to carry the *aod-4* gene, for open reading frames (ORFs) and restriction sites. The remaining 79 cosmids and 11 BACs, which cover about 600 kbp of supercontig 3 distal to the *al-3* sequence, have yet to be analyzed and isolated. The sequence analysis revealed several enzymes that could be used to insure that each predicted ORF (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003) for the sequence was present on at least two distinct restriction fragments no larger than 10 kbp. No striking candidate genes were recognized when these ORFs were examined for similarity to known signaling components. By far the most common characterization of these ORFs was as a hypothetical or predicted protein (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003). The cosmids and BACs were grown individually and then combined into pools of 5 to 10 clones. DNA was isolated from these pools of clones, digested with the appropriate restriction enzymes, and used in transformation experiments. Since this approach was labour intensive, I also attempted transformation of sphaeroplasts with uncut pools of cosmid and BAC DNA.

Preliminary assays for rescue of growth in the presence of antimycin A of the *aod-4* strain EN195-109 by transformation with either cut or uncut pools of the BACs and cosmids from the 2 Mbp region of the right arm of linkage group V have been unsuccessful. A number of possibilities could explain the lack of success. The *aod-4* gene could lie on the region of supercontig 3 that has not yet been tested. It is also conceivable that the *aod-4* gene lies in one of the gaps between supercontigs or in one of the areas that is not represented by the available libraries. Another possibility is that single clones from the tested region could have been under-represented when the pools were isolated. I am currently isolating each BAC and cosmid individually for use in transformation experiments to rule out the latter possibility.

The *chl-1* gene was also mapped and found to be on the right arm of linkage group II near the *nuc-2* gene (see section 3.14; Table 14; Fig. 24B). The *chl-1* gene is most likely between the *arg-5* and *arg-12* markers. This region is covered by portions of supercontigs 4 and 20, which consist of 1.9 Mbp of sequence (Fig. 24B; assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003). Again, there is a gap of unknown size between these supercontigs. There are cosmids and BACs available for supercontig 4, but approximately 200 kbp of sequence between *arg-5* and the centromere is not covered by clones in any available library. This lack of coverage is most likely because supercontig 20 contains centromeric sequences that were covered by random clones during the shotgun sequencing (Galagan *et al.*, 2003). Since the *chl-1* mutant strains can grow in the presence of antimycin A, transformants cannot be selected based on rescue of growth as in the case of rescuing the *aod-4* mutation. Therefore, I have used individual cosmids from the region near the *nuc-2* marker for transformation of the *chl-1* strain EN294-46. Individual transformants were tested for alternative respiration when grown in the presence of chloramphenicol. No transformants have been obtained that have restored the ability of the strain to induce alternative oxidase activity in the presence of chloramphenicol. The possibility that the *chl-1* mutation cannot be rescued because it is dominant to the wild-type version of the gene is ruled out by the complementation analysis, which showed that *chl-1* strains were complemented by a number of other strains to give chloramphenicol inducible alternative oxidase activity (see section 3.10).

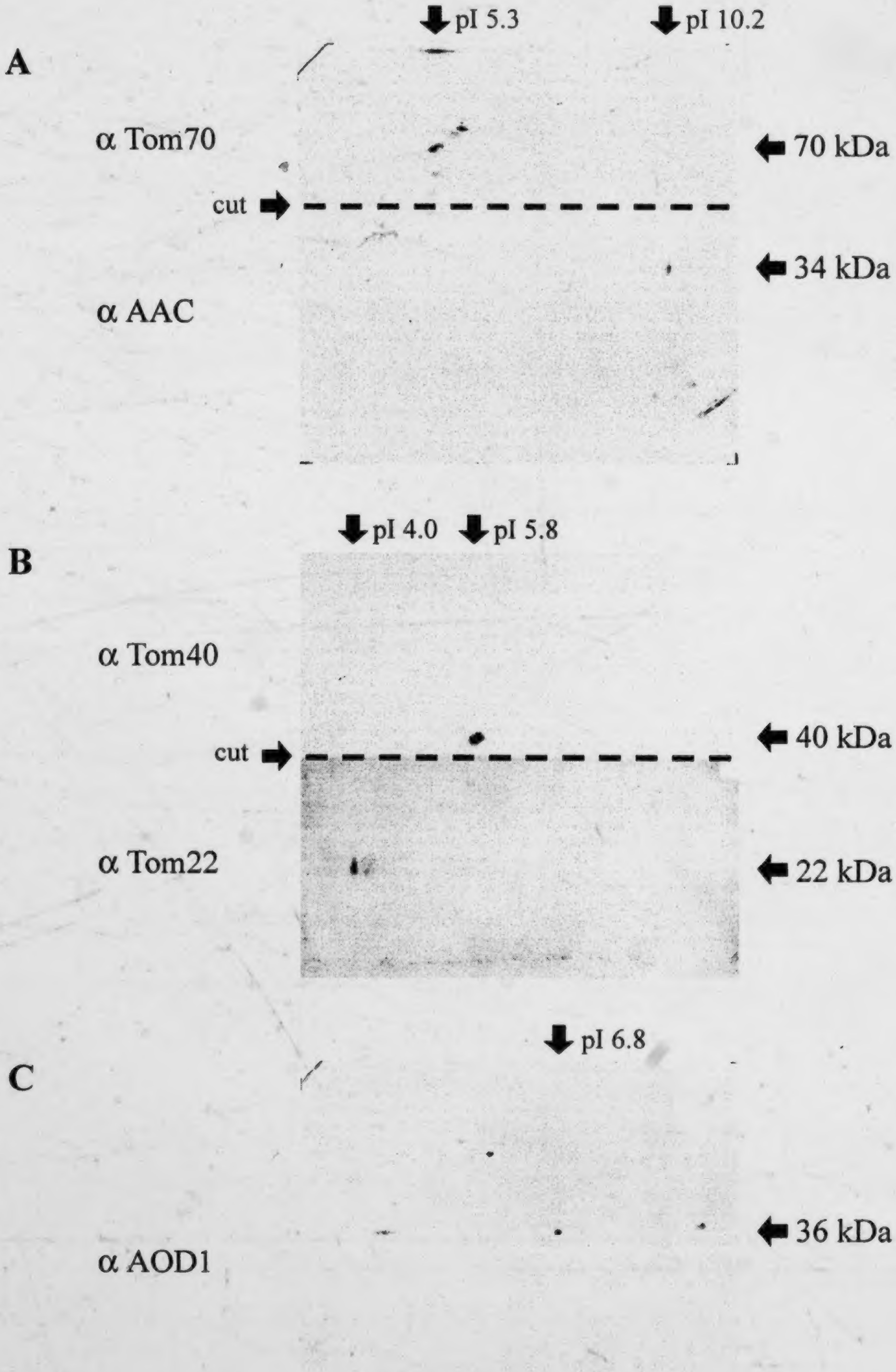
I have also been involved in several attempts to rescue the *aod-2* mutation. The *aod-2* complementation group was mapped to linkage group II, showing 7% recombination with the *arg-5* marker and 16% recombination with the *thr-3* marker (Bertrand *et al.*, 1983; H. Bertrand, personal communication). These markers also surround the centromere, and the *aod-2* gene is believed to be located between the *arg-5* marker and the centromere (H. Bertrand, personal communication). As mentioned above, the centromeric region of linkage group II is not well covered by available genomic libraries. Nevertheless, cosmids and BACs that have been located in this region (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003), and that are available, have been isolated and used to transform an *aod-2* mutant strain. None of the transformants isolated and tested to date have chloramphenicol inducible alternative respiration or the capability for growth in the presence of antimycin A (data not shown; T. Aziz, C. Nargang and F. Nargang, personal communication). The *aod-2* gene could be in a region near the centromere that is not available in the genomic libraries or that has not yet been successfully sequenced and placed in the *N. crassa* genome sequence due to the highly repetitive nature of the DNA in this region.

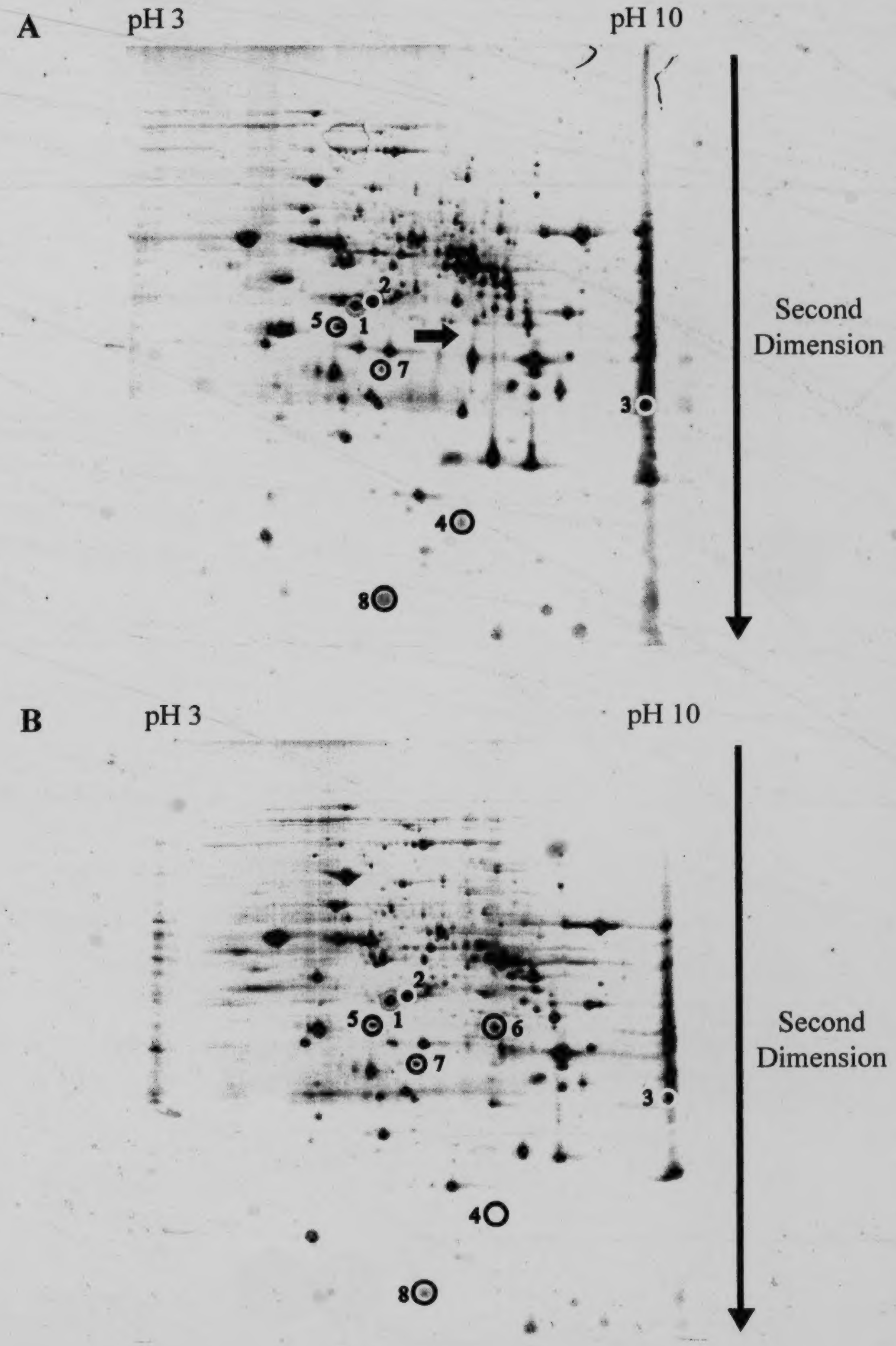
3.16 Two-Dimensional gel electrophoresis

Although it was clear from this study and previous studies (see section 1.9; Edwards *et al.*, 1976; Bertrand *et al.*, 1983) that alternative oxidase regulatory mutations can be isolated, it is unknown if any of these mutations have more global effects on mitochondrial biogenesis in addition to, or rather than, specific effects on alternative oxidase regulation. To address this possibility, I wished to analyze mitochondria isolated from non-induced and chloramphenicol induced cultures of various strains by 2-D gel electrophoresis to see if the mitochondrial proteome was significantly altered.

It was possible that highly hydrophobic mitochondrial membrane proteins would not be solubilized during the preparation of mitochondrial proteins for 2-D gel analysis (Berkelman and Stenstedt, 1998). To insure that mitochondrial membrane proteins entered the first dimension, non-SDS containing gel, I transferred 2-D gels of mitochondrial proteins from wild-type strain NCN246 to nitrocellulose and decorated the blot with antiserum to the outer membrane proteins Tom70, Tom40, Tom22, and the inner membrane protein AAC (Fig. 25). Tom40 is an integral β-barrel protein of the MOM (see section 1.4; Truscott *et al.*, 2003; Rehling *et al.*, 2003), while AAC is a polytopic integral protein of the MIM (Saraste and Walker, 1982). Tom70 is anchored to the MOM by an N-terminal membrane spanning domain (McBride *et al.*, 1992), while Tom22 is anchored to the MOM via a C-terminal membrane spanning domain (Rodriguez-Cousiño *et al.*, 1998). All of these proteins were detected by immunoblotting (Fig 25, A and B). Thus, it seemed likely that if these proteins were solubilized and entered the gel, then most other membrane proteins would as well.

The identities of the Tom40 and AAC proteins were further confirmed by picking the identified protein spots from silver stained gels, and obtaining amino acid sequence data for these spots using MALDI-TOF mass spectrometry (Fig. 26, spots 5 and 3, respectively). Using a similar approach of Western blotting (Fig. 25C) and MALDI-TOF sequencing (Fig. 26, spot 6), I also identified the AOD1 protein from a gel of mitochondrial proteins isolated from a chloramphenicol induced culture of NCN246. The AOD1 protein was not detected by immunoblotting of a gel of mitochondrial proteins from a non-induced culture of NCN246 (data not shown). From visual inspection, the alternative oxidase protein spot was not present on silver stained gels containing


A
pI 5.3
pI 10.2
α Tom70
70 kDa
cut
34 kDa
α AAC
B
pI 4.0
pI 5.8
α Tom40
40 kDa
cut
α Tom22
22 kDa
C
pI 6.8
36 kDa
α AOD1


A
pH 3
pH 10
2
5
1
7
3
4
8
Second Dimension
B
pH 3
pH 10
2
5
1
6
7
3
4
8
Second Dimension

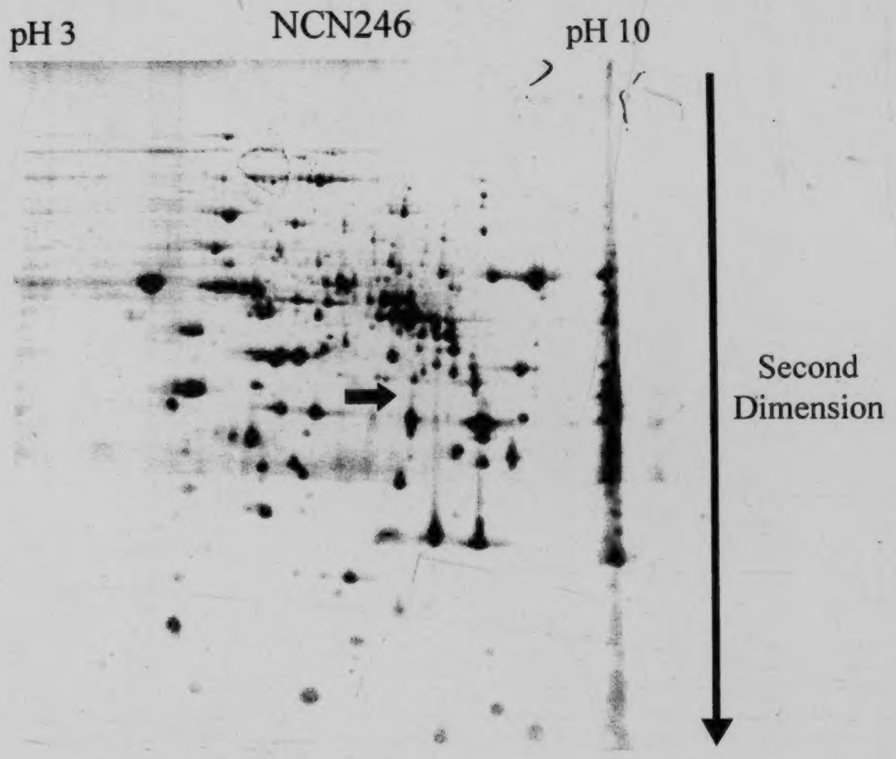
mitochondrial proteins from non-induced cultures of alternative oxidase wild-type strains NCN246, T1P11, and T11-76 (Fig. 27, indicated with an arrow). Five other protein spots were also isolated for sequence determination (Fig. 26). Two of these spots (Fig. 26, spots 1 and 2) were chosen because they were spatially close to the area in which I believed Tom40 would be located from Western analysis, and were thus candidate spots for the Tom40 protein (Fig. 25). The other three spots (Fig. 26, 4,7 and 8) were chosen because of apparent differences in the amounts of these proteins between the non-induced and chloramphenicol induced cultures (Fig. 26; Fig. 27; see below).

Since it appeared that the 2-D gel technique would allow visualization and identification of proteins from *N. crassa* mitochondria, I proceeded with this analysis. The three alternative oxidase wild-type strains NCN246, T1P11 and T11-76 were analyzed, and will eventually serve as controls for all the isolated mutant strains. I chose to analyze three phenotypically different mutant strains: an *aod-1* mutant strain, an *aod-2* mutant strain, and the *chl-1* mutant strain that exhibited a resistance to chloramphenicol. Visual inspection of the gels from non-induced cultures of strains NCN246, T1P11, T11-76, *aod-1*, *aod-2*, and *chl-1* revealed no obvious differences (Fig. 27). The gels of the chloramphenicol induced samples from these strains are also visually indistinguishable except that the gels from *aod-1*, *aod-2*, and the *chl-1* strain do not have AOD1 protein (Fig. 27). Though no gross alterations were observed, changes in poorly resolved proteins or weakly stained spots would not be identified by visual inspection.

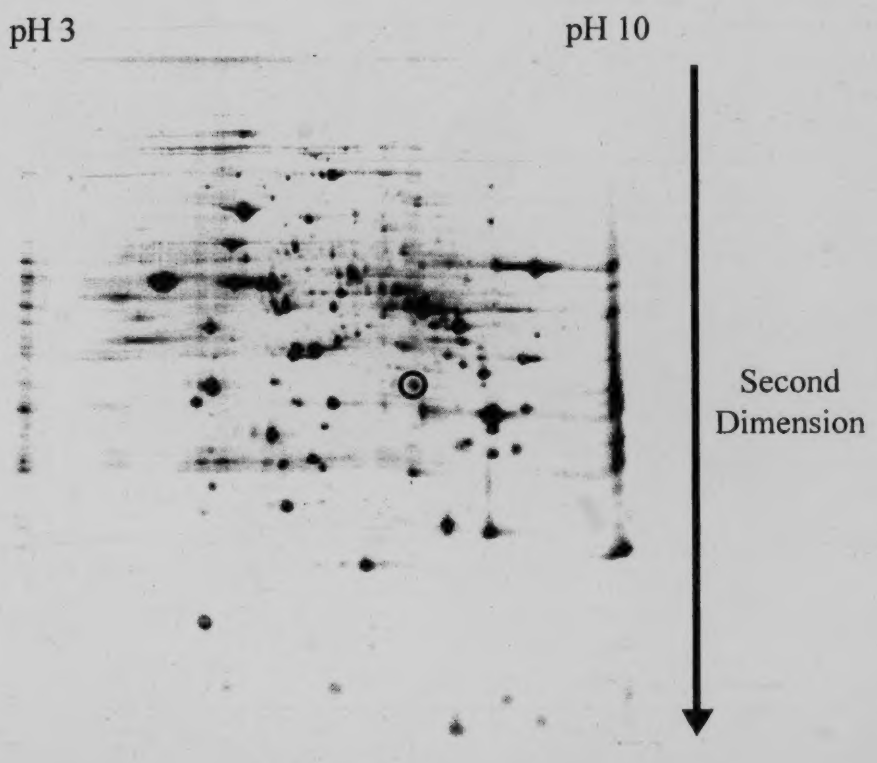
On the other hand, comparison of silver stained gels between non-induced and chloramphenicol induced cultures did reveal that growth in the presence of chloramphenicol resulted in several obvious differences in mitochondrial proteins in both wild-type and mutant strains (Fig. 27). Sequencing information obtained using MALDI-TOF analysis was performed to identify spots that were obviously different between the two growth conditions. Other protein spots that differ in intensity between the two growth conditions were detected but were not analyzed here. The amounts of others may also vary but were not identified by visual inspection.

Two protein spots appeared darker in the non-induced samples compared to the chloramphenicol induced samples of all the strains analyzed (Fig. 26, spots 4 and 8; Fig. 27). One of these is the HEX-1 protein (Fig. 26, spot 4) of *N. crassa*, which is a major

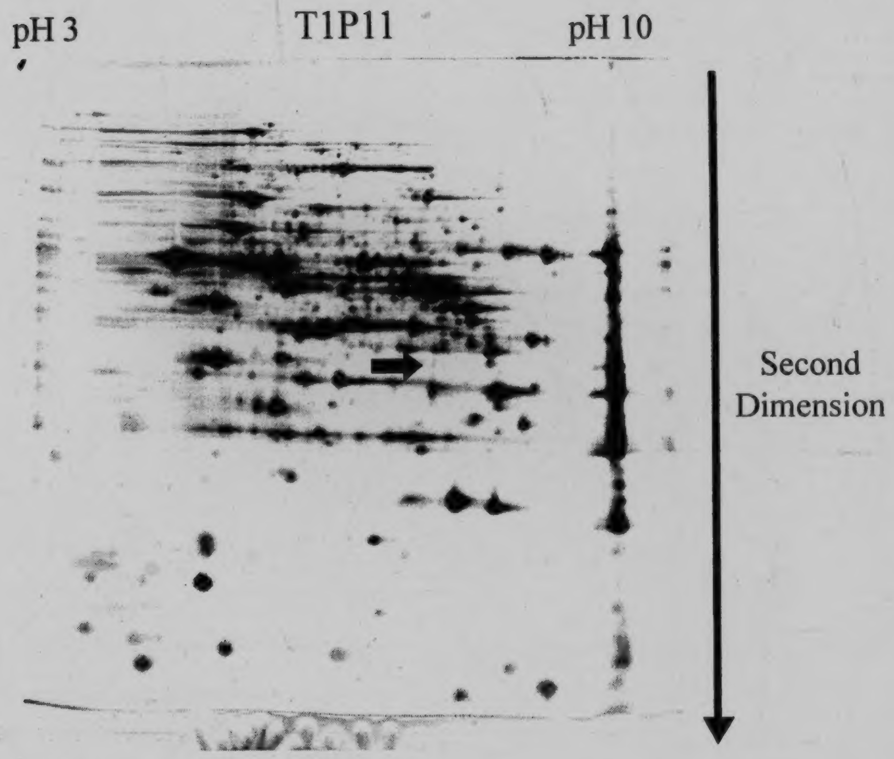
A


pH 3
NCN246
pH 10
Second Dimension

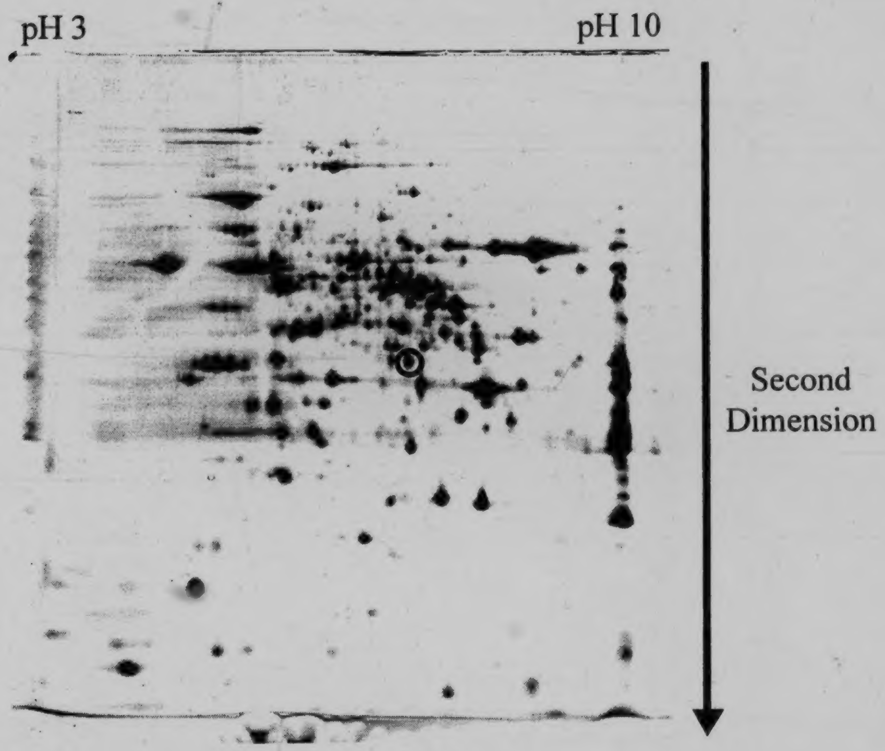
B

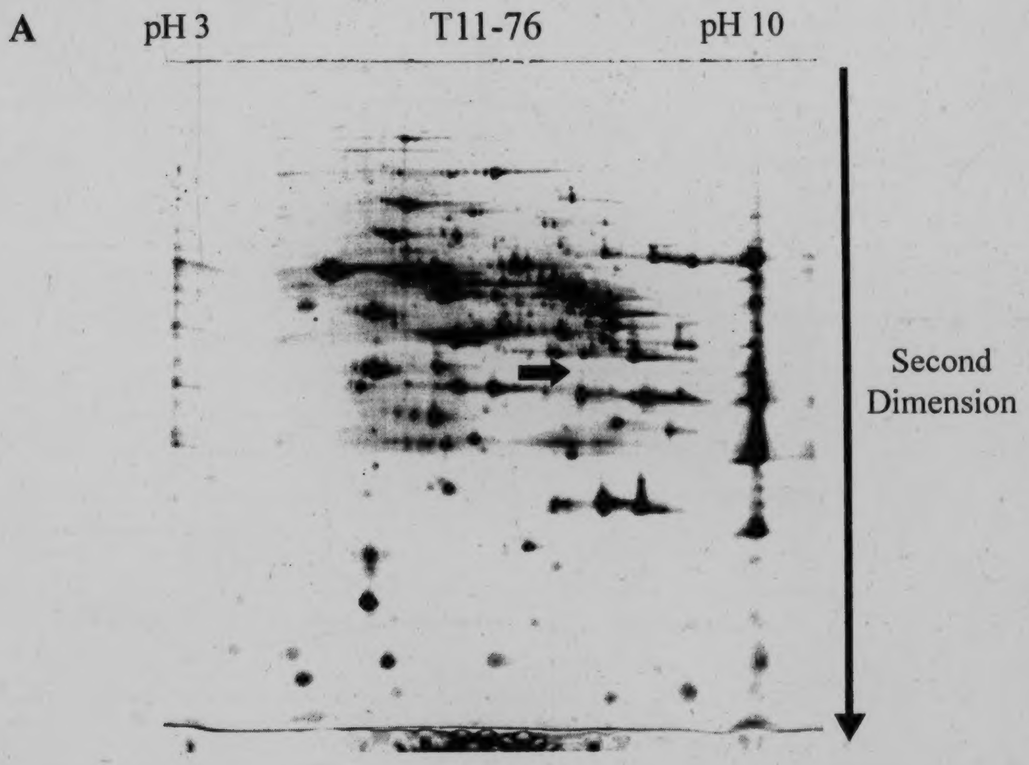

pH 3
pH 10
Second Dimension

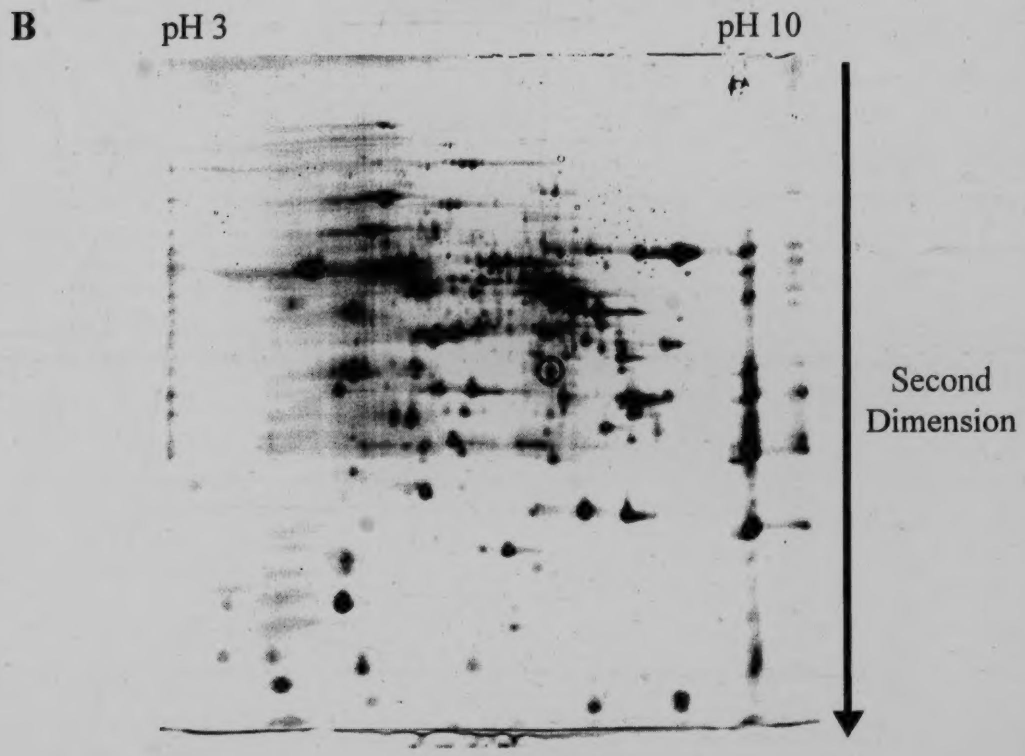
A

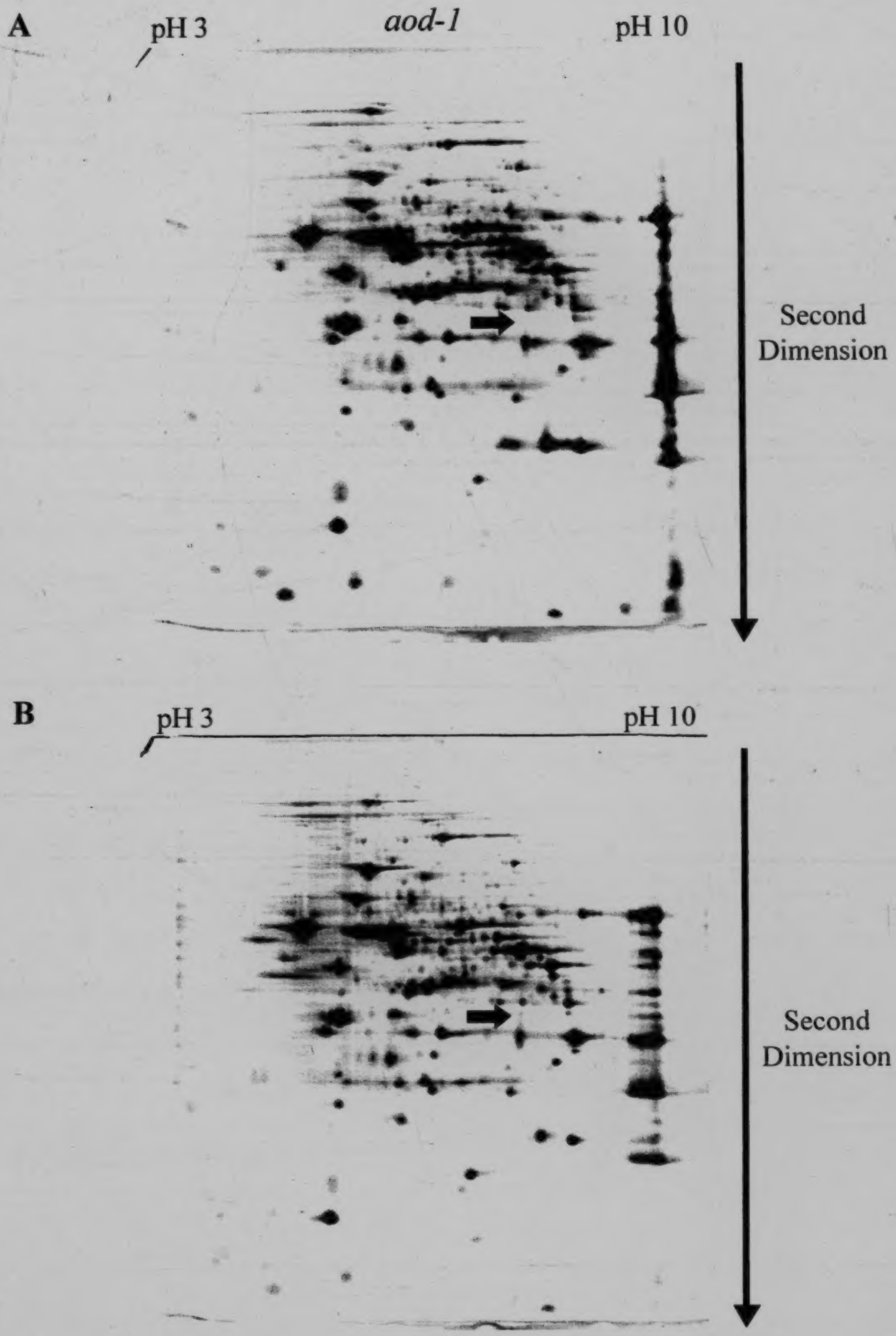

pH 3
T1P11
pH 10
Second Dimension

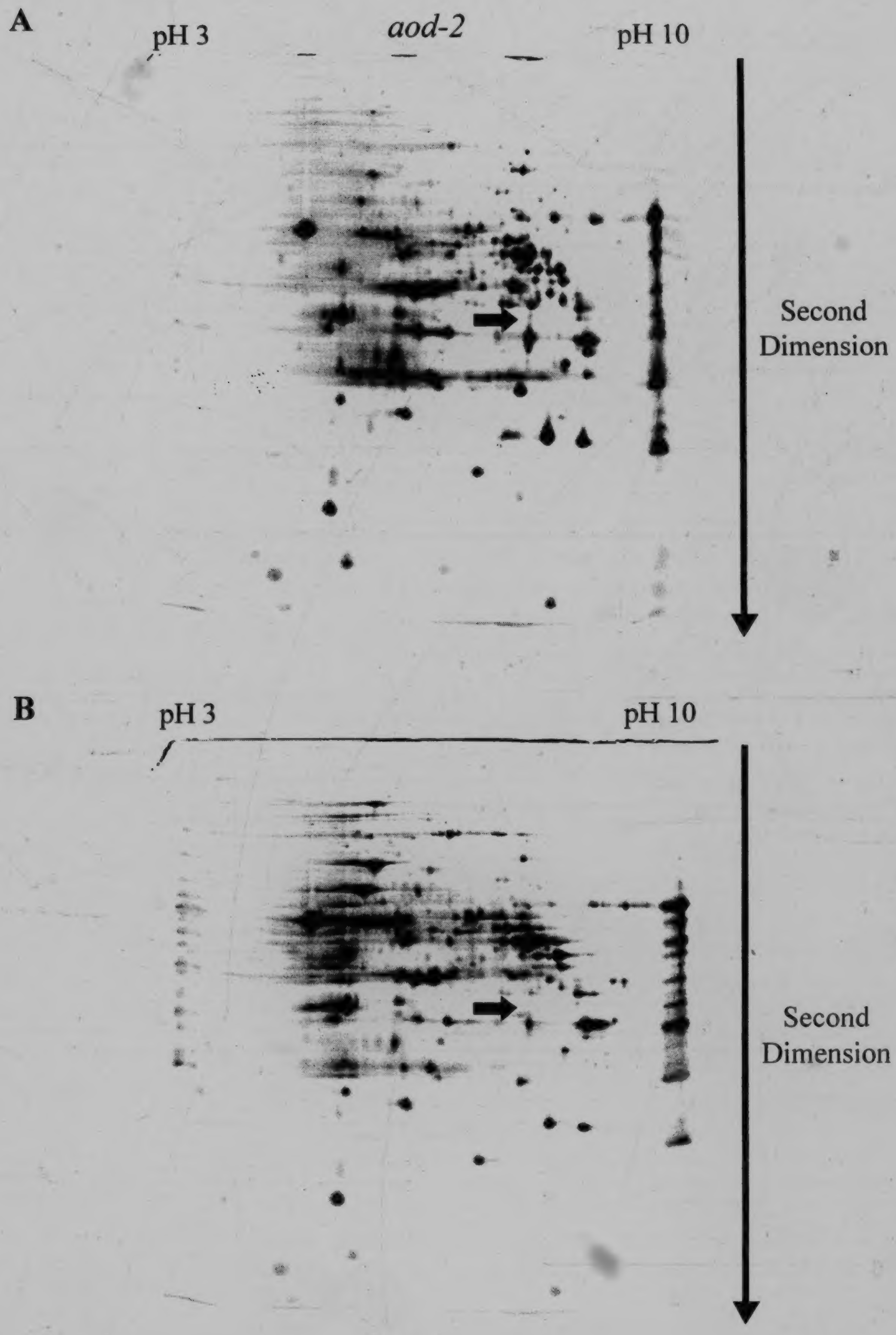
B

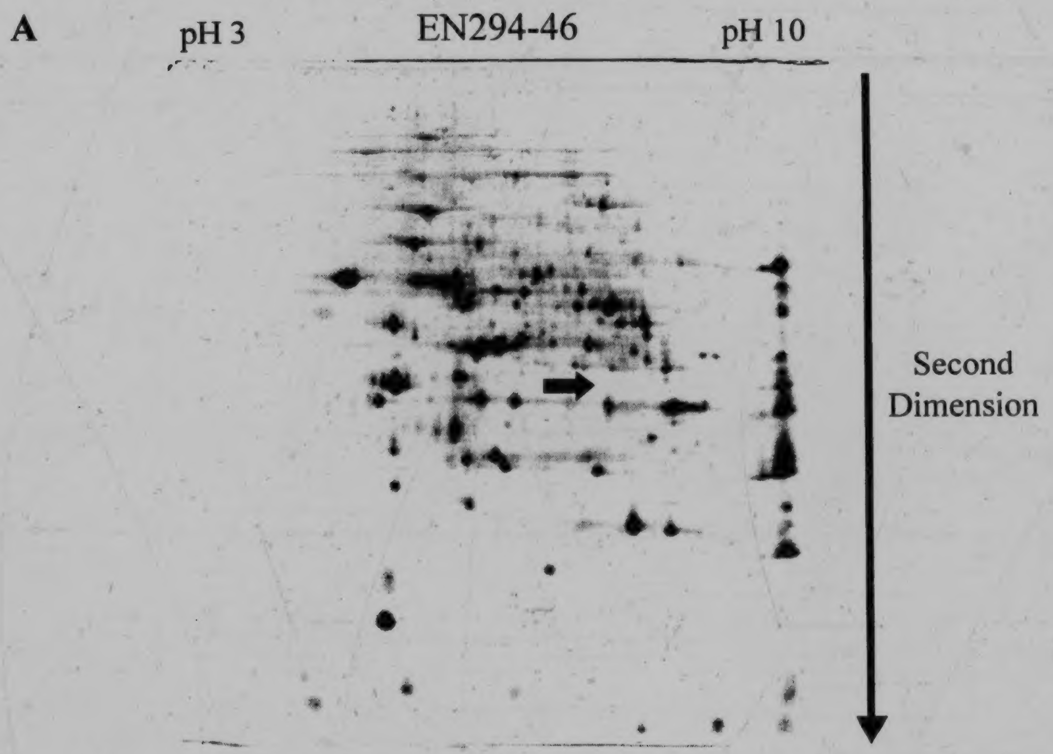

pH 3
pH 10
Second Dimension

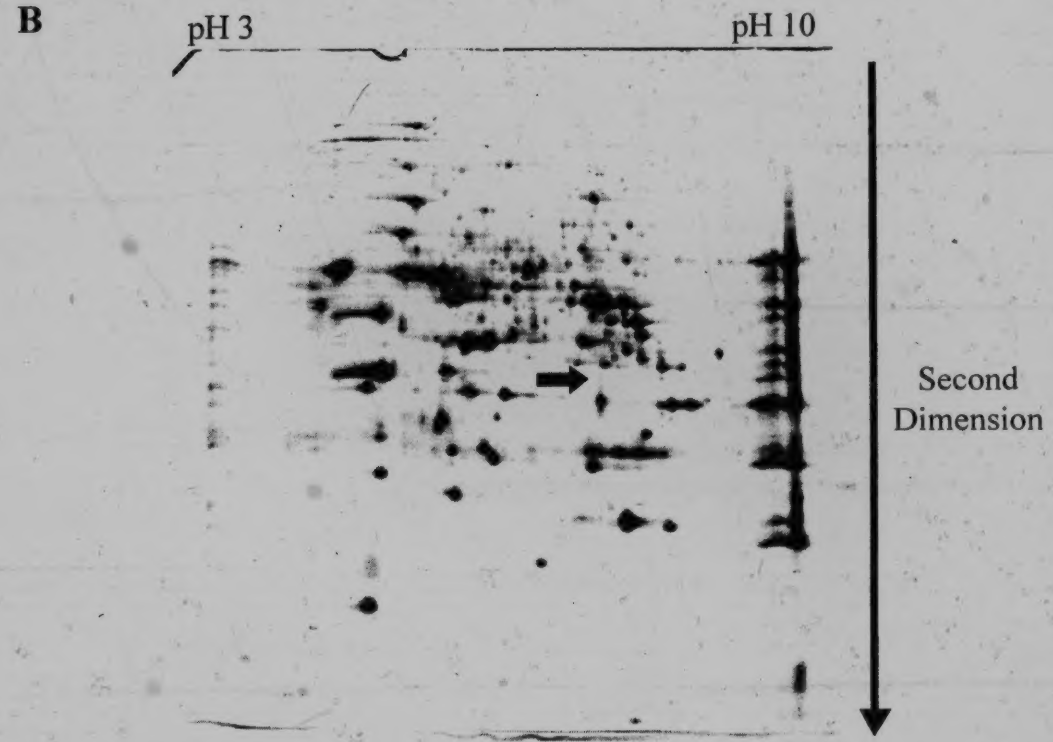

A
pH 3
T11-76
pH 10
Second Dimension


B
pH 3
pH 10
Second Dimension


A
pH 3
aod-1
pH 10
Second Dimension
B
pH 3
pH 10
Second Dimension


A
pH 3
aod-2
pH 10
Second Dimension
B
pH 3
pH 10
Second Dimension


A
pH 3
EN294-46
pH 10
Second Dimension


B
pH 3
pH 10
Second Dimension

component of Woronin bodies that plug septal openings between cells when hyphae are damaged to prevent the catastrophic loss of cytoplasm from the syncytium (Tenney *et al.*, 2000; Jedd and Chua, 2000). The HEX-1 protein has a peroxisomal targeting signal and fractionates with these dense organelles, not mitochondria. The reproducible presence of this protein in the purified mitochondrial preparations could be a contaminant or it could indicate that HEX-1 also localizes to mitochondria. It is unclear why levels of this protein would be reduced by growth in the presence of chloramphenicol.

Another protein identified was comparatively darker in the non-induced gels of all the strains examined (Fig. 26, spot 8; Fig. 27). This spot was shown to have sequence similarity to a ribonucleotide-diphosphate reductase small chain from *Streptomyces lipmanii* (Borovok *et al.*, 2002). Ribonucleotide reductases accept electrons from thioredoxin or glutaredoxin at a diferric center, and use these electrons to reduce ribonucleotides to deoxyribonucleotides (Jordan and Reichard, 1998; Borovok *et al.*, 2002). It is interesting that conditions that induce alternative oxidase, an enzyme that has been theorized to provide protection against ROS (see section 1.8), results in a reduced amount of a protein that uses the electrons carried by two proteins also involved in protection from ROS (Carmel-Harel and Storz, 2000). Conceivably, ribonucleotide reductase levels could be reduced to allow for an increase in the ROS-protective activities of thioredoxin and glutaredoxin.

A third spot is darker under inducing conditions compared to non-inducing conditions for all the strains examined (Fig. 26, spot 7; Fig. 27). This spot was shown to have sequence similarity to an alcohol dehydrogenase from *Xanthomonas axonopodis* (da Silva *et al.*, 2002) and a glutathione-S-transferase-like protein from *Galleria mellonella* (Li *et al.*, 2002). Glutathione-S-transferases in plants have been shown to be induced by oxidative stress and may catalyze the addition of glutathione to ROS damaged metabolites (Edwards *et al.*, 2000; Marrs, 2002). If this protein is a glutathione-S-transferase, then it could be upregulated in response to increased levels of ROS when chloramphenicol is present.

4 Discussion and Future Work

4.1 Role of the upstream CRE in regulating alternative oxidase

CREs are *cis*-acting regulatory sequences found in a variety of organisms that respond to cAMP levels in the cell and control the expression of a variety of genes (Habener *et al.*, 1995; Montminy, 1997). Both the human MnSOD (Kim *et al.*, 1999) and cytochrome *c* (Gopalakrishnan and Scarpulla, 1994; Herzig *et al.*, 2000) genes contain CREs in their promoters. Activation of the CRE binding protein (CREB) causes increased transcription of these genes. Thus, CREs are known to be involved in regulating the expression of nuclear encoded mitochondrial proteins. Mitochondrial dysfunction in human cell cultures resulting from loss of mtDNA, or inhibition with a variety of drugs such as antimycin A, oligomycin (a complex V inhibitor) or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; an uncoupler of hydrogen ion pumping to electron flow) has been shown to cause phosphorylation of a CREB (Arnould *et al.*, 2002). The phospho-CREB was then able to localize to the nucleus and drive transcription of the CRE regulated c-*fos* gene and a reporter system consisting of several CRE sites upstream of a luciferase gene. CREs are also known to be used as regulatory elements in *N. crassa* since the circadian rhythm regulated, glucose repressible gene *ccg-1*/ *grg-1* contains an upstream CRE that regulates its transcription (Wang *et al.*, 1994).

The upstream region of the *N. crassa aod-1* gene contains a consensus CRE sequence (Li *et al.*, 1996), as do the alternative oxidase genes from *A. nidulans*, *Gelasinospora spp.* (Tanton *et al.*, 2003) and *M. grisea* (Yukioka *et al.*, 1998a). We were interested in determining if the *N. crassa aod-1* CRE was similarly involved in regulating gene expression.

When this project was begun, gel retardation assays had recently revealed that a protein bound to the *aod-1* CRE of *N. crassa* (now published in Tanton *et al.*, 2003). In an attempt to isolate the CRE binding protein, I tried to develop a yeast one hybrid screen. However, a reporter construct carrying four tandem copies of the CRE upstream of the yeast *HIS3* gene showed such high levels of leaky reporter expression when introduced into the yeast strain that the screen could not be done. It is likely that *S. cerevisiae* contains a protein that bound the *N. crassa* CRE sequence and induced expression of the reporter. For instance, the *S. cerevisiae* CREB protein Sko1p has been shown to bind a CRE motif (Nehlin *et al.*, 1992) and could have bound our reporter.

Because of the possible role of the CRE in regulating *aod-1* expression, I also characterized the respiration of a number strains of *N. crassa* with mutations known to affect cAMP signaling pathway(s). The colonial temperature sensitive gene *cot-1* is a member of the PKA family and was required for regulation of hyphal elongation, possibly in response to blue light (Yarden *et al.*, 1992; Lauter *et al.*, 1998). Shifting a temperature sensitive allele of *cot-1* to the restrictive temperature caused cells to develop abnormal mitochondrial morphologies (Gorovits *et al.*, 2000). The cAMP dependent protein kinase *cpk* mutation caused PKA kinase activity to become cAMP independent (Murayama *et al.*, 1985). The microcycle blastoconidiation *mcb* gene has homology to a regulatory subunit of PKA and a mutant strain showed a loss of growth polarity (Bruno *et al.*, 1996). Finally, the adenylate cyclase *cr-1* mutant strain made little to no endogenous cAMP (Pall *et al.*, 1981). I tested each of these strains for alternative oxidase activity in the presence and absence of chloramphenicol and found that none of the cAMP signaling pathway mutations affected regulation of alternative oxidase. With respect to *cr-1*, my results concur with a previous study, which also showed that induction of alternative oxidase is unaffected in a *cr-1* mutant strain (Pall *et al.*, 1981). Thus, none of these known components of cAMP signaling pathways affect the regulation of *aod-1* in *N. crassa*.

Later studies in the lab eventually showed that deletion of the CRE did not affect alternative oxidase production under non-inducing conditions or in response to induction by chloramphenicol (Tanton *et al.*, 2003). Thus, the CRE is not required for regulation of *aod-1* expression in response to growth in the presence of chloramphenicol. It is possible that the CRE may play a role in regulation of *aod-1* transcription in response to other signals that could result from unknown stresses or specific effects on individual oxidative phosphorylation components.

4.2 Possible involvement of two-component or retrograde signaling pathways

As discussed in section 1.10.1, regulation of the expression of nuclear encoded mitochondrial proteins could involve a two-component signaling pathway similar to the stress response two-component pathways found in bacterial and other fungal species. The *N. crassa os-1/nik-1* gene was isolated based on homology to the histidine kinase two-component osmosensors of bacteria and yeast (Alex *et al.*, 1996; Schumacher *et al.*, 1997). Mutant strains failed to properly assemble the cell wall and were therefore unable to adapt to increased osmotic pressure. Despite the homology of the histidine kinase domains of *S. cerevisiae sln1* and *N. crassa os-1*, *os-1* was not predicted to have membrane spanning domains. Instead *os-1* had an N-terminal hydrophobic region and may therefore be targeted to the ER or to mitochondria (Schumacher *et al.*, 1997). I tested the alternative oxidase activity of an *os-1* mutant strain to determine if this two-component histidine kinase was involved in the signaling pathway responsible for *aod-1* induction. I found that respiration of the *os-1* mutant strain was indistinguishable from wild-type following growth under non-inducing conditions or in the presence of chloramphenicol.

It is also possible that *N. crassa* contains a signaling pathway with homology to the retrograde regulation pathway of *S. cerevisiae* (see section 1.10.3) that modulates nuclear gene expression in response to mitochondrial dysfunction. Using a BLAST search engine, I compared the Rtg1p, Rtg2p and Rtg3p amino acid sequences to the *N. crassa* database (assembly version 2, Galagan *et al.*, 2003) and retrieved a putative *RTG2* orthologue. The *N. crassa rtg-2* gene was mutagenized through the process of RIP, and *rtg-2* mutant strains were obtained (M. Chae and F. Nargang, personal communications). Preliminary results suggest that regulation of alternative oxidase activity in these strains is unaffected.

4.3 Tyrosinase reporter system

Previous attempts to identify *aod-1* regulatory components had shown a bias towards isolation of mutations in the alternative oxidase structural gene (see section 1.11). To avoid this partiality, I developed a reporter system using a 3.3 kbp upstream region of *aod-1* fused to the coding sequence for the enzyme tyrosinase. The tyrosinase reporter construct pBAT was introduced into strain T1P11, producing the reporter strain T11-76. Growth of this strain in the presence or absence of chloramphenicol clearly demonstrated that expression of the tyrosinase reporter was controlled in a manner similar to *aod-1* gene expression. Colonies of the reporter strains grown in the presence of chloramphenicol, an inducer of *aod-1* gene expression, turned brown within five hours of the addition of the tyrosine solution, while colonies grown under non-inducing conditions remained white. This was the first successful reporter system designed in the lab, and the ability of this reporter to mimic *aod-1* induction was the first step in delineating the functional *aod-1* promoter, as the 3.3 kbp upstream region cloned into pBAT contains the necessary *cis*-acting regulatory sequences responsible for *aod-1* induction by chloramphenicol.

Later work revealed that 250 bp of *aod-1* upstream sequence were sufficient to regulate chloramphenicol induction of an *aod-1*$^{+}$ transgene in an *aod-1* mutant background (Tanton *et al.*, 2003). The entire *aod-1* promoter region could be limited to this 250 bp region. Alternatively, it is possible that further upstream sequences, perhaps including the CRE, are required for regulation of *aod-1* gene expression in response to other treatments or conditions. A new tyrosinase reporter strain with a construct carrying only these 250 bp fused to the tyrosinase coding sequence could be made. This strain, the reporter strain T11-76, and the *aod-1* mutant strain carrying the transgene with only 250 bp of upstream sequence could all be compared for induction of alternative oxidase activity and/or reporter activity during growth in the presence of inhibitors that block electron flow at specific complexes or other stresses, such as temperature, hydrogen peroxide, or different carbon sources. Conceivably, expression of *aod-1* in response to failure of distinct complexes or other stresses could occur through the same *cis*-acting regulatory site(s) as chloramphenicol induction, or through distinct sequences. Mutations in nuclear encoded proteins involved in oxidative phosphorylation could also be crossed into these strains to see if they affect transgenic *aod-1* or reporter gene expression. It is interesting to note that while the *aod-2* mutant strain does not induce *aod-1* expression in response to chloramphenicol or antimycin A, overgrowth of the strain in the presence of chloramphenicol can eventually cause the appearance of cyanide-insensitive respiration (H. Bertrand, personal communication; data not shown). This induction might be construed as evidence that overgrowth of the fungus stimulates another regulatory pathway that is able to induce *aod-1* expression, and perhaps this pathway acts via a different regulatory pathway than induction by chloramphenicol.

4.4 Characterization of the tyrosinase RIP allele

Early in this study, considerable confusion resulted from the use of the T1P11P08 and T1P11P13 strains in the tyrosinase plate assay because they usually remained white during the first hours of the assay, but often turned brown within 1 day of the addition of exogenous tyrosine. At first I believed that this colour change was due to leaky expression from the RIPed *T* gene. However, the rate of colour change was similar to that seen for the T^+ strain NCN10, suggesting that the level of this putative leaky expression was about the same as levels of endogenous expression. These ambiguous observations, together with the inability to isolate mutants using these strains with the reporter construct, led me to characterize the supposedly RIPed *T* gene in strains T1P11, T1P11P08 and T1P11P13.

Strain T1P11 was the direct result of a cross designed mutate the *T* gene by RIP, and strains T1P11P08 and T1P11P13 were progeny of a T1P11 back-cross (S. Free, personal communication). Therefore, all three strains should have carried the same RIP mutations. However, sequencing revealed that only strain T1P11 carries a RIPed allele of the *T* gene.

The sequenced region of the T1P11 *T* gene had 89 characteristic RIP base transition mutations that caused 39 missense and 3 nonsense changes to the predicted tyrosinase amino acid sequence. The first non-sense codon truncated the 407 amino acid mature tyrosinase protein to only 72 amino acids. Since this truncation removes one of the conserved copper binding domains (Seo *et al.*, 2003), this mutation most likely destroys tyrosinase activity. In addition, genes affected by RIP are often not transcribed (Rountree and Selker, 1997), so it is unlikely that the RIPed *T* gene in T1P11 is expressed. The T1P11P08 and T1P11P13 *T* sequences were identical with each other and showed no evidence of RIP. Apparently these strains did not inherit the RIPed copy of *T* from T1P11, but rather inherited a T^+ allele from the other parent strain.

The sequences obtained for the strains were compared with those published for strains OR (74-OR8-1*a*; FGSC #988) and TS (69-1113; FGSC #2963; Kupper *et al.*, 1989), and for strain 74-OR23-1V*A* (FGSC #2489) published on the Whitehead database (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003). The

T1P11P08, T1P11P13, and Whitehead sequences had identity with the OR sequence at seven of eight polymorphic sites between strains OR and TS, while T1P11 had identity with the OR sequence at five of the polymorphic sites. The nucleotide sequence at these polymorphic sites supports the idea that T1P11P08 and T1P11P13 inherited the *T* allele from the wild-type parent during the back-cross rather than from T1P11. This notion could be confirmed if the *T* gene from the wild-type parent that was originally crossed to T1P11 were to be sequenced and compared to T1P11 and the offspring.

The T1P11, T1P11P08, T1P11P13, and Whitehead DNA sequences all contained a novel polymorphism at base +1327 that did not alter the amino acid sequence of the protein. The reported nucleotide sequences at this position were not polymorphic between strains OR and TS (Kupper *et al.*, 1989). The T1P11, T1P11P08, T1P11P13, and Whitehead DNA sequences also contained a single base insertion at + 2248 relative to the original OR and TS reported sequences that changed the reading frame, replacing the last 26 amino acids of the published TS and OR sequences with 90 amino acids. It is unlikely that the two differences are due to sequencing errors reported in this thesis since they are present in all three of the strains that I sequenced and in the Whitehead sequence. The polymorphic site and the insertion could be natural variants of the *T* gene, or the previously published sequences for strains OR and TS could contain sequencing errors. The insertion occurs after the C-terminal site of cleavage for tyrosinase maturation and would not affect the amino acid sequence of the mature protein. Indeed, the amino acid sequence of the mature protein was determined by analysis of chemical cleavage products (Lerch, 1978; Lerch *et al.*, 1982), and this sequence correlated with the amino acid sequence predicted from the *T* gene sequence (Kupper *et al.*, 1989). The antibody developed against the C-terminal region (Kupper *et al.*, 1990b) would still be expected to recognize a 183 amino acid portion of the C-terminal cleaved region that occurs before the site of insertion. The predicted molecular weight for the published OR and TS protyrosinase polypeptides was 68.6 kDa, while the predicted molecular weight for the translation product of the Whitehead sequence with the single base insertion was 75.8 kDa. Western blotting revealed tyrosinase bands approximately 75, 52 and 46 kDa in size (Kupper *et al.*, 1990b), and *in vitro* translation of the *T* mRNA gave a single translation product of about 75 kDa in weight (Kupper *et al.*, 1989). These observations support the suggestion that the C-terminus of protyrosinase does indeed contain the novel 90 amino acids rather than the published 26 amino acids. This suggestion could be confirmed by directly sequencing the 75kDa tyrosinase precursor polypeptide.

4.5 Analysis of the mutagenic approaches

Two methods were used to generate mutant strains that did not properly induce alternative oxidase. The first method involved directly plating the mutagenized conidia of the reporter strain on chloramphenicol containing media, while the second included an enrichment for mutant conidia by selecting for a lack of growth in antimycin A. The two methods yielded 18 mutant strains, 6 from the first method and 12 from the second.

A comparison of the two methods revealed that the first was approximately 5000 fold more effective than the second in producing isolated mutant strains from a given number of conidia treated (see section 3.8.1; Table 9). The first method yielded 6 mutant strains out of 183, 500 conidia treated or about 3×10^{-3} %, while the second method generated 12 mutants out of 1.8×10^{9} condia treated or about 7×10^{-7} %. One possible reason for this relatively large difference in effectiveness is that conidia containing *aod-1* regulatory mutations could have been lost in the second approach during the filtration step, which removes any growing, germinating conidia. Ungerminated conidia could have been caught in growing and branching hyphal strands extending from other conidia and removed from the culture inadvertently. Recessive mutations could also be lost if mutant conidia fused with wild-type conidia, creating a heterokaryon that could properly induce alternative oxidase and grow despite the presence of antimycin A. These fusion events are minimized by agitation of the culture (Davis, 2000), but the four days of growth in liquid culture could give an increased opportunity for heterokaryon formation. In addition, it is possible that *aod-1* regulatory mutant conidia did not survive the four days in antimycin A containing media, as a loss of viability over an enriching starvation period can occur (Davis, 2000).

Although the first method is more effective at generating *aod-1* regulatory mutant strains from a given number of EMS treated conidia, the second method allows a larger number of EMS treated conidia to be screened with much less effort. On average, an evaluation of the colour of colonies from a tyrosinase plate assay took eight hours when screening the large numbers of colonies derived using the first method, but less than one hour when screening the small numbers of colonies remaining after the enrichment of the second method. Thus, the second approach was less labour-intensive because the majority of the EMS treated conidia, which would not have contained a mutation affecting reporter expression, were able to grow in antimycin A containing media and were removed by filtration.

The second method also involved picking fewer colonies, as 8% of the initially treated conidia were picked as white colonies when the first method was used while only 6×10^{-6} % of the treated conidia were picked with the second method. Therefore, fewer strains had to be re-tested with the tyrosinase plate assay and characterized for their respiratory phenotype. Using the second method, we picked less than one tenth the number of white colonies picked during screening with the first method, and yet isolated double the number of mutant strains. Overall, the likelihood of isolating an *aod-1* regulatory mutant strain from a white colony picked during the initial tyrosinase plate assay was about 27 fold higher with the second method than the first. This increase in efficiency must be due to the filtration enrichment step.

In addition, a number of false positive strains that remained white during the plate assay but did not contain a mutation affecting the regulation of *aod-1* were found using both methods. However, fewer white colonies and fewer false positives were isolated with the second approach as compared to the first. Of 183, 500 conidia screened with the first method, 140 (8×10^{-2} %) were false positives, while of 1.8×10^{9} conidia screened with the second method, 30 (2×10^{-6} %) were false positives. Thus, the second method was 40, 000 fold more effective in removing false positive strains. As expected, the enrichment method removed false positive strains, as these strains would be able to properly induce alternative oxidase in antimycin A containing media and grow.

For both methods combined there were a total of 170 strains that had been picked based on the initial white colony colour, remained white during the subsequent second tyrosinase plate assay, but were able to produce cyanide-insensitive respiration when grown in the presence of chloramphenicol. These false positives from our mutagenesis screen could be the results of mutations to the integrated reporter construct itself. Mutations in the reporter *T* gene or the 3.3 kbp *aod-1* upstream region could affect inducible tyrosinase activity, preventing the colour change from occurring during the plate assay. It is unlikely, however, that all 170 strains contain mutations to the reporter construct. Other possibilities include mutations to factors involved in protyrosinase maturation, or in the uptake of tyrosine, although such factors have not yet been identified.

4.6 Isolation of alternative oxidase regulatory mutants

Using the first mutagenic method, six putative *aod-1* regulatory mutants were isolated: *E1*, *E2*, *E3*, *E4*, *E7* and *E15*. Mutations *E1* and *E15* represented two new complementation groups called *aod-4* and *aod-7* that were involved in the regulation of *aod-1* (this study; I. Cleary and F. Nargang, personal communication). Mutation *E2* was the only allele of the *chl-1* gene isolated and appears to be the first chloramphenicol resistant mutation isolated in *N. crassa* (Perkins *et al.*, 2001). Mutation *E3* seemed to be an allele of *aod-2* from complementation and mapping studies. The second method generated twelve putative *aod-1* regulatory mutants: *E5*, *E6*, *E8*, *E9*, *E10*, *E11*, *E12*, *E13*, *E14*, *E16*, *E17*, and *E18*. While mutations *E8*, *E9*, *E11*, *E13*, and *E14* were all allelic to the *E1* mutation and fell into the *aod-4* complementation group, mutations *E10* and *E12* represented two more complementation groups involved in *aod-1* regulation called *aod-5* and *aod-6* (this study; I. Cleary and F. Nargang, personal communication). Mutations *E4*, *E5*, *E6*, *E7*, *E16*, *E17*, and *E18* have not yet been fully characterized.

In crosses of the mutant strains to various auxotrophic and mapping strains, the regulatory mutations were all inherited in a Mendelian fashion, demonstrating that the mutations are in nuclear encoded genes. Ideally, the mutant strains obtained would have been back-crossed several times to a wild-type strain before characterization insure that the effects on alternative oxidase regulation were due to a single mutation. This would have also minimized the possibility that the mutant strains carried secondary mutations that could have affected growth, cytochrome spectra, or other characteristics of each strain. However, time constraints made more than one or two back-crosses impractical. Strains carrying each of the characterized mutations were able to complement strains with mutations from other complementation groups, demonstrating that none of the mutations were dominant.

In total, four novel complementation groups that regulate *aod-1* expression have been isolated by our mutagenesis and selection schemes. Both approaches resulted in the isolation of a similar number of novel complementation groups, although the second method clearly showed a bias towards isolation of mutations in *aod-4*. Only the *aod-4* complementation group had multiple alleles isolated, suggesting that our screen for *aod-1* regulatory components has not been saturated. Perhaps the *aod-4* locus is a hotspot for EMS mutagenesis. The other regulatory loci may also have partial redundancy with one another, *aod-2*, or other as yet unidentified loci, such that only certain mutations within these genes that have a drastic effect on gene function would alter *aod-1* regulation. Another possibility is that *aod-4* mutations are more tolerable in the fungus because the *aod-4* gene product is relatively less important for overall function and survival. The other gene products could be essential, so that the mutations that were isolated at a comparatively low frequency at these loci might have only partially affected gene function, allowing the strains to survive but perturbing *aod-1* regulation. Characterization of the gene products of the loci that were mutagenized may provide insight into why the *aod-4* complementation group has been isolated with a relatively high frequency.

Previous studies using UV radiation (Edwards *et al.*, 1976) or *N*- methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Bertrand *et al.*, 1983) with subsequent incubation in liquid media containing antimycin A to enrich for alternative oxidase mutants failed to identify the four new regulatory genes described in the present study. The first screen produced six regulatory mutations, three of which were placed in the *aod-1* complementation group (Edwards *et al.*, 1976; Bertrand *et al.*, 1983). Perhaps the other three mutations belong to one or more of the four complementation groups affecting alternative oxidase expression that we have isolated. The mutagenic agent used may also have affected the bias of mutations that were obtained. UV radiation can cause single base pair changes, frameshifts of one to several base pairs, deletions, or chromosomal rearrangements in *N. crassa* (Davis and de Serres, 1970). MNNG causes transition mutations, predominantly AT to GC substitutions, while EMS causes both transition mutations, predominantly GC to AT, and frameshift mutations. The use of EMS instead of UV or MNNG could possibly have facilitated the mutagenesis of our novel regulatory genes.

In addition, the first method involving directly plating EMS treated conidia may have allowed isolation of mutants that were not obtainable in the previous screens. Plating of the conidia immediately after mutagenesis may have allowed strains that would have died in liquid media due to an intolerance of starvation over the enrichment time (see section 4.5) to instead survive on solid media. The second method described in this thesis was based on removal of conidia able to grow in the presence of antimycin A by filtration. However, the original screens utilized inositoless death as an enrichment technique, whereby inositol requiring cells growing and dividing in media lacking inositol burst due to an inability to correctly synthesize the cell wall (Edwards *et al.*, 1976; Bertrand *et al.*, 1983). Mutagenesis of this strain and incubation in media lacking inositol and containing antimycin A caused those cells able to induce alternative oxidase to grow and burst, leaving only the conidia unable to induce alternative oxidase activity. Perhaps the *inl* strain was more sensitive than strain T11-76 to the effects of the mutations that I have isolated and could not survive carrying mutant alleles at these novel loci.

Despite the possibilities listed above, the most likely reason for our isolation of additional alternative oxidase regulatory mutants is the use of the reporter system. Previous methods that relied on a loss of alternative oxidase function clearly led to a bias towards mutations of the structural gene. Perhaps these *aod-1* mutations caused a stronger phenotype than the regulatory mutations and so were isolated with higher frequency.

4.7 Growth rates of isolated mutant strains

Analysis of the growth rates revealed that growth of the *aod-4* mutant strain EN195-109 on non-inducing media at 30°C reduced the growth rate compared to alternative oxidase wild-type strains. Strangely, the growth rate at 30°C was not further reduced by the addition of chloramphenicol, such that the strain grew at a similar rate both with and without chloramphenicol. However, this delayed growth was not seen at room temperature, where growth of EN195-109 on non-inducing media was similar to the rates of the control strains and faster than growth in the presence of chloramphenicol. The *E1* mutation could produce a temperature sensitive effect, causing the *aod-4* gene product to be less functional at 30°C than at room temperature. However, temperature sensitivity would not explain why the effect of increasing the temperature of growth and adding the inhibitor chloramphenicol were not additive, and there was no further reduction in the strain's growth rate in the presence of chloramphenicol at 30°C. Perhaps the effects of the *E1* mutation at 30°C mimic some of the effects of growth in the presence of chloramphenicol so that there is no additive effect. The reduced growth rate in non-inducing conditions could be due to decreased levels of cytochromes aa_3 and *b* (see section 4.8). However, if this were the case, then the further reduction in the levels of these cytochromes that was seen when the strain was grown in the presence of chloramphenicol would be expected to further reduce the growth rate. A trivial explanation for the inability to see a difference in growth rate between non-induced and chloramphenicol induced cultures could be that the race tubes only measure the rate of elongation and not mycelial mass. Thus, the mass of the culture in the non-induced race tube could be more than that in the chloramphenicol containing race tube. This hypothesis could be tested by obtaining growth data from the mass of mycelia generated during growth of liquid cultures. Other mutations of the *aod-4* complementation group have not been tested for growth rate, though it is known that these strains were also deficient in cytochromes aa_3 and *b* following growth at 30°C (see section 4.8).

Strains carrying the *chl-1 E2* allele had reduced growth rates at both 30°C and room temperature under all conditions compared to the control strains. This reduced rate could be due to the *E2* mutation itself or to some secondary mutation(s) at other loci. However, comparison of the growth rate of *E2* mutant strain EN294-46 with its *E2* mutant progeny strain E2a-119 revealed that both strains had a reduced growth rate (data not shown). Both strains also exhibited a severely reduced growth rate compared to the initial growth rate after about 7 days of growth at room temperature in the presence of chloramphenicol. It is difficult to explain why a mutation that imparts resistance to the effects of chloramphenicol would also lead to reduced viability in its presence. Perhaps the procedure through which these mutant stains provide resistance to chloramphenicol causes a toxic byproduct to be generated or exhausts the cellular supply of a metabolite, which could lead to a decrease in viability over time.

4.8 Cytochrome spectra of isolated mutant strains

Cytochrome spectra for the mutant strains were obtained from cultures grown at 30°C. The non-induced culture of the *aod-4* strain EN195-109 (carrying the *E1* allele) contained reduced levels of cytochromes aa_3 and *b* as compared to the other strains, though the levels were higher than in control strains grown in the presence of chloramphenicol. The reduced levels of these cytochromes may explain the reduced growth rate of strain EN195-109 relative to control strains (see section 4.7). Other strains in the *aod-4* complementation group, including the *E8*, *E9*, *E11*, and *E14* alleles, also showed this reduction in levels of cytochromes aa_3 and *b* under non-inducing conditions (data not shown; I. Cleary and F. Nargang, personal communication). Thus, the decrease in these cytochromes in the *E1* mutant is not allele specific, but occurs in other isolated *aod-4* mutant strains. The *aod-7* mutant strain also exhibited reduced levels of cytochromes aa_3 and *b* in non-inducing conditions, while strains carrying mutations in the *aod-1*, *aod-2*, *aod-5* or *aod-6* complementation groups had wild-type spectra (see section 3.12; I. Cleary and F. Nargang, personal communication). The decrease in cytochromes suggests that the mutations in the *aod-4* gene may not only affect alternative oxidase regulation, but may also affect gene products required for proper synthesis or assembly of the cytochrome respiratory pathway.

The strain carrying the *E2* mutation was the only strain in which growth in the presence of chloramphenicol did not affect the cytochrome spectra. In addition, this strain did not induce alternative oxidase in the presence chloramphenicol, but did in the presence of antimycin A. Taken together, these observations led to the conclusion that this mutation causes the strain to be chloramphenicol resistant.

4.9 The chloramphenicol resistance gene *chl-1*

As described above, the *E2* mutation was the only mutation isolated that showed differential expression of *aod-1* in response to chloramphenicol versus antimycin A and has been classified as a chloramphenicol resistant mutation. Since the levels of cytochromes aa_3 and *b* were not reduced when this strain was grown in chloramphenicol, there was likely no respiratory deficiency under this condition, which would explain why alternative oxidase was not induced by this treatment. Furthermore, the strain was able to induce alternative oxidase in response to growth in the presence of antimycin A. Strains carrying the *E2* mutation were able to complement all the strains tested, so *E2* is the only isolated mutation of this complementation group. To date, there are no known nuclear or mitochondrial encoded chloramphenicol resistance markers in *N. crassa* (J. Chalmers and D. Perkins, personal communication).

mtDNA mutations have been isolated that cause chloramphenicol resistance in *A. nidulans* (Gunatilleke *et al.*, 1975), *P. anserina* (Belcour and Begel, 1977), and *S. cerevisiae* (Waxman *et al.*, 1979). Chloramphenicol inhibits bacterial and mitochondrial translation by binding to the ribosome and preventing the association of tRNA with the A site, thereby blocking peptidyl transfer (Schlünzen *et al.*, 2001). These fungal chloramphenicol resistant mitochondrial mutations could affect the rRNA genes encoded in the mtDNA. Mutations in the 23S rRNA gene of *E. coli* (Vester and Garrett, 1988) or of *Halobacterium halobium* (Mankin and Garrett, 1991), or in the mtDNA encoded 16S rRNA gene of mice (Howell and Lee, 1989) have been shown to cause chloramphenicol resistance. However, the *E2* mutation is in a nuclear encoded gene because it segregated in a Mendelian fashion.

Chloramphenicol resistance in several bacterial species has also been shown to be due to chloramphenicol efflux pumps, phosphotransferases, or acetyltransferases (Schlünzen *et al.*, 2001). Chloramphenicol producing *Streptomyces venezuelae* uses a chloramphenicol 3'-*O*-phosphotransferase to modify and inactivate chloramphenicol (Mosher *et al.*, 1995; Izard and Ellis, 2000; Izard, 2001). Many other bacterial species use a chloramphenicol acetyltransferase to inactivate chloramphenicol (Murray and Shaw, 1997). *Pseudomonas aeruginosa* and other bacterial contain three-component multidrug efflux pumps that can export chloramphenicol (Bissonnette *et al.*, 1991; Hancock and Speert, 2000). Interestingly, expression of a glucose transporter in the fungus *C. albicans* is linked to drug resistance (Varma *et al.*, 2000). The *E2* mutation could be involved in activating one of these systems in *N. crassa*.

4.10 Alternative oxidase expression

Previous Northern analysis has revealed that induction of alternative oxidase activity in wild-type cells by growth in the presence of chloramphenicol or antimycin A caused an increase in the steady state levels of *aod-1* mRNA (Li *et al.*, 1996; Tanton *et al.*, 2003; this study). Nuclear run-on assays showed that non-induced wild-type cultures constitutively expressed *aod-1* at a low level, and treatment with antimycin A caused an increase in the amount of transcription (Tanton *et al.*, 2003). Thus, regulation of *aod-1* expression under normal conditions and in response to inducing treatments occurs at the level of the transcript, through increased transcription of the gene and possibly increased stability of the mRNA. Northern blots of non-induced cultures usually show little to no *aod-1* mRNA accumulation, and Western blots of these non-induced cultures show no protein (Li *et al.*, 1996; Tanton *et al.*, 2003; this study). The lack of AOD1 protein in non-induced cultures suggests that *aod-1* regulation also occurs post-transcriptionally, by reducing the stability of the small amount of constitutively expressed mRNA, and/or by preventing its translation.

Unexpectedly, we found that non-induced cultures of the *T* mutant strain T1P11 and the reporter strain T11-76 contain variable steady state levels of *aod-1* mRNA. Unusually high levels of *aod-1* mRNA have been seen occasionally in uninduced cultures of other alternative oxidase wild-type strains (Tanton *et al.*, 2003) but never as consistently as in strains T1P11 and T11-76. In all cases where *aod-1* mRNA has been found in non-induced cultures, no alternative oxidase activity nor AOD1 protein has been detected (this study; Tanton *et al.*, 2003). Again, this suggests that *aod-1* expression is regulated post-transcriptionally, and directly implies control at the translational level.

Strains T1P11 and T11-76 could have a higher level of constitutive *aod-1* transcription than wild-type. Alternatively, these strains may have a reduced rate of degradation that allows the steady state *aod-1* mRNA level to become higher than in other alternative oxidase wild-type strains. The possibility that the accumulation of mRNA in non-induced cultures is due to the tyrosinase null mutation has been ruled out by demonstrating that the *T* mutation does not co-segregate with the accumulation of *aod-1* mRNA (K. Lam and F. Nargang, personal communication). Therefore, an unknown gene is responsible for this phenotype.

The aberrant accumulation of *aod-1* mRNA in these strains had not been discovered before we decided to use the tyrosinase reporter system. Although we have been able to isolate regulatory mutations using these strains, it is possible that the background of constitutively high accumulation of *aod-1* mRNA could have precluded the isolation of weaker regulatory mutations. That is, a weak mutation could theoretically cause an inability to induce alternative oxidase expression in a different strain, but if the reason for accumulation of *aod-1* mRNA in the background and reporter strains is due to increased transcription, a weak regulatory mutation might not have a pronounced affect on induction. On the other hand, we have observed no *aod-1* transcript in the *aod-4*, *aod-5* and *aod-7* mutant strains isolated using the T11-76 reporter system (see section 3.13; I. Cleary and F. Nargang, personal communication). Thus, the mutants either prevent *aod-1* transcription or increase the level of an *aod-1* mRNA degradation system under both non-inducing and inducing conditions. Nuclear run-on assays of the alternative oxidase regulatory mutant strains grown under non-inducing and inducing conditions could determine if the effects are at the transcriptional or post-transcriptional level. Nevertheless, mutations affecting alternative oxidase expression have been isolated, which was the desired result at the outset of the study. The accumulation of *aod-1* mRNA in the reporter strain under non-inducing conditions has not precluded the goal of this study from being reached.

4.11 Mapping and rescue of isolated mutations

Genetic mapping placed mutations *E1* and *E11* from the *aod-4* complementation group on the right arm of linkage group V, with 1-35% recombination with the marker *inl*. Recombination frequencies for different regions of the genome have been reported to vary up to ten fold if the crosses involved used strains with different genetic backgrounds (Perkins and Bojko, 1992; Davis, 2000). Several genes in the *uvs-6* epistasis group are involved in the recombinational repair system in *N. crassa*, and mutations in some of these genes, such as *mei-2* and *mei-3*, can reduce meiotic recombination and increase the frequency of chromosomal non-disjunction (Perkins *et al.*, 2001). However, the decrease in recombination frequency seen is accompanied by a high percentage of inviable and white spores from the cross. Aside from crosses involving the *alcoy* strain, which contains multiple chromosomal translocations, I did not observe a high proportion of inviable spores during the mapping crosses. Thus, it is unlikely that different alleles of genes involved in genome wide recombination caused the wide range in recombination frequencies seen in the mapping crosses for the *E1* and *E11* mutations.

Several genes and *cis*-acting sequences have been implicated in affecting recombination at specific loci or regions of the *N. crassa* genome (Catcheside and Corcoran, 1973; Catcheside, 1974b; Catcheside, 1986). Different alleles of these genes and sequences have been found in different natural isolates of *N. crassa*. Inheritance of different alleles of the *rec-1* gene caused an eight fold variation in recombination frequency between alleles of *nit-2* (linkage group I; Catcheside, 1970), up to a thirty fold range in recombination frequency between alleles of *his-1* (linkage group V; Jessop and Catcheside, 1965; Catcheside and Austin, 1969), and a two fold variation in recombination frequency between *asp* and *ad-7* (linkage group V; Catcheside, 1974a). Similarly, the *rec-2* gene affected the recombination frequency between alleles of *his-3* by five fold (linkage group I; Angel *et al.*, 1970), between *arg-3* and *sn* by two fold (linkage group I; Catcheside and Corcoran, 1973), between *his-3* and *ad-3A* by four fold (linkage group I; Catcheside, 1979), and between *pyr-3* and *leu-2* by up to ten fold (linkage group IV; Catcheside and Corcoran, 1973). The *rec-3* gene affected the recombination frequency between alleles of *his-2* by eight fold, between *sn* and *his-2* by twenty fold (linkage group I; Catcheside and Corcoran, 1973), and two different alleles of *rec-3* affected recombination frequency at the *am* locus up to twenty-five fold (linkage group V; Catcheside 1975). The *cis*-acting sequence *cog* modulated the effect of *rec-2* on recombination between *his-3* alleles by six fold, which meant that together, *rec-2* and *cog* affected recombination in this region by about 30 fold (Angel *et al.*, 1970). Heterozygosity between the strains in the cross at another *cis*-acting region, *ss*, affected recombination between alleles of *nit-2* between two and twenty fold (Catcheside, 1981). In combination, the *ss* sequence and *rec-1* affected recombination in this region by about 100 fold. Unfortunately, not all lab stock strains have been characterized for their alleles at the *rec* loci, nor we do not know the genetic background of a number of the strains that were used for mapping the mutations. However, clearly there are recombinational control systems that are operating in the region in which the *E1* and *E11* mutations map that cause large variations in recombinational frequency.

By mapping the *E1* and *E11* mutations to the right arm of linkage group V, I have narrowed the search for the *aod-4* gene and the attempts to rescue the *E1* mutation have been limited to those genomic DNA library clones with sequences specific to this region. Continuing attempts at rescuing the ability of an *E1* strain to grow in the presence of antimycin A involve transforming the strain with cosmid and BAC clones from three supercontigs localized to this region that cover about 2.6 Mbp of sequence (assembly version 3, *Neurospora* genome sequence, Galagan *et al.*, 2003). Thus, mapping these mutations has reduced the search for *aod-4* from over 40 Mbp of estimated total genomic sequence (Galagan *et al.*, 2003) to 2.6 Mb. Unfortunately, the sequence and clone availability for this region is not contiguous, with two gaps of unknown size between the supercontigs and about 36 kbp of sequence not present in available genomic clones. It is possible that the *aod-4* gene lies in one of these gaps or unavailable regions, in which case the rescue would be unsuccessful. Another update of the *N. crassa* sequencing project could provide us with the clone sequence information to cover these regions.

The *E2* mutation from the *chl-1* complementation group was mapped to the right arm of linkage group II and most likely lies within the markers *arg-5* and *arg-12*, with 3% recombination obtained with the marker *nuc-2*. Again, the sequence information for this region is not contiguous, with a gap of unknown size between two supercontigs that cover 1.9 Mbp of this region. Selection for rescue of the *chl-1* mutation is more difficult than for *aod-4* because the *chl-1* mutant strain is able to grow in the presence of antimycin A. Nevertheless, a *chl-1* mutant strain has been transformed with cosmids near the *nuc-2* marker and transformants have been assayed for the induction of alternative oxidase in the presence of chloramphenicol. Identification of a novel *N. crassa* antibiotic resistance gene would be valuable for use as a resistance marker in this fungus.

Current mapping in the lab has placed the *aod-5* gene on linkage group VI, and the *aod-7* gene on linkage group IV, most likely on the left arm (I. Cleary and F. Nargang, personal communication). Initial mapping of the *aod-6* gene with the *alcoy* strain did not reveal any linkage to the *alcoy* markers. Thus, the *aod-6* gene could be on linkage group VII, which is not part of the translocations that make up the *alcoy* strain, or it could be fairly distal on one of the arms of any other linkage group as these regions can still recombine in an *alcoy* cross (Perkins *et al.*, 1969; Davis, 2000). Taken together, these mapping data show that each of the newly isolated complementation groups is at a distinct location of the genome from one another, and from *aod-1* and *aod-2*. This effectively rules out any possibility that intra-allelic complementation caused an over-estimation of the number of new alternative oxidase regulatory genes identified.

4.12 Mitochondrial proteome analysis

Mitochondrial proteins from NCN246, T1P11, T11-76, *aod-1*, *aod-2*, and *chl-1* strains grown in the presence or absence of chloramphenicol were analyzed by two-dimensional electrophoresis to reveal if any of these mutations dramatically affected the mitochondrial proteome. At the outset of these experiments, we were concerned that hydrophobic mitochondrial membrane proteins, which represent a sizable proportion of the proteome, might not be released from the MOM and MIM, and would not be present on the gels. To determine if these proteins would enter the gel, samples from wild-type strain NCN246 were subjected to 2-D electrophoresis and Western blots for the MOM proteins Tom70, Tom40 and Tom22, and the MIM proteins AOD1, and AAC were analyzed. All these proteins were detected on the blot, and the corresponding spots for Tom40, AAC, and AOD1 on another set of gels were picked and sequenced. MALDI-TOF sequencing confirmed that 2-D gels did contain these membrane proteins.

Comparison of the gels from the mutant strains *aod-1*, *aod-2*, and *chl-1* (EN294-46) with the gels from wild-type strain NCN246, *T* mutant strain T1P11, and reporter strain T11-76 showed that the mutations were not associated with global changes of the mitochondrial proteome that could be detected by visual inspection. As expected, all of the non-induced strains and the induced mutant strains did not contain the protein identified as AOD-1 from Western analysis. We are awaiting the establishment of a differential fluorescent staining system for co-separating samples in the department (Ettan DIGE system from Amersham Pharmacia Biotech, San Francisco, CA) to optimize reproducibility and quantification. Computer analysis of such an analysis may reveal more data than visual inspection.

I did notice that for all the strains examined, certain spots were reproducibly different between all non-induced and all chloramphenicol induced samples. Two spots that appeared darker in non-induced samples than in induced samples were picked for

MALDI-TOF sequencing. One is the previously identified *N. crassa* HEX-1 protein (Tenney *et al.*, 2000; Jedd and Chua, 2000). The *hex-1* gene encodes the major protein of Woronin bodies, which plug septal pores between cells when hyphae are damaged to prevent the loss of cytoplasm from the syncytium. The HEX-1 protein has a peroxisomal targeting signal and fractionates with these organelles. The reproducible presence of this protein in the purified mitochondrial preparations from non-induced cells could be a contaminant from damaging the cells during harvesting, or it could indicate that HEX-1 also localizes to mitochondria. It is unclear why levels of this protein would be reduced by treatment with chloramphenicol. Perhaps growth in the presence of chloramphenicol has a secondary effect that reduces hyphal damage that occurs during growth.

The second spot has homology to the small subunit of the ribonucleotide-diphosphate reductase of *Streptomyces lipmanii* (Borovok *et al.*, 2002). Ribonucleotide reductases use electrons gained from thioredoxin or glutaredoxin to catalyze the reduction of ribonucleotides to deoxyribonucleotides (Jordan and Reichard, 1998; Borovok *et al.*, 2002). Thioredoxin and glutaredoxin are involved in protection against ROS (Carmel-Harel and Storz, 2000). Perhaps the level of ribonucleotide reductase is reduced in chloramphenicol induced cells to allow these two enzymes to use their electrons for ROS reduction. Treatment with chloramphenicol could cause an increase in ROS by impairing cytochrome mediated respiration. In response, the cell induces expression of alternative oxidase, which has been implicated to play a protective role against reactive oxygen species (see sections 1.7.5 and 1.8). At the same time, the cell could decrease expression of the ribonucleotide reductase to allow thioredoxin and glutaredoxin to reduce ROS.

A third spot was picked for sequencing because it was darker under inducing conditions than non-inducing conditions in all samples. The sequence obtained has homology to an alcohol dehydrogenase from *Xanthomonas axonopodis* (da Silva *et al.*, 2002) and a glutathione-S-transferase-like protein from *Galleria mellonella* (Li *et al.*, 2002). In plants, oxidative stress induces glutathione-S-transferases, which are then thought to add glutathione to ROS damaged metabolites (Edwards *et al.*, 2000; Marrs, 2002). An increase in ROS due to growth in the presence of chloramphenicol could result in an upregulation of a glutathione-S-transferase to deal with the cellular damage.

4.13 Summary

Apart from the previously identified *aod-2* mutation and the uncharacterized ANT-2, ANT-3, and ANT-5 mutations (Edwards *et al.*, 1976), the mutations obtained during this study represent the only regulatory mutations isolated for induction of alternative oxidase in any organism. Four novel complementation groups have been identified and implicated in alternative oxidase regulation. Thus, there is at least one regulatory pathway involving several individual components that is responsible for inducing expression of a nuclear encoded gene in response to mitochondrial dysfunction. Further study of these mutations will provide insight into the nature of this pathway, and whether it functions solely to affect alternative oxidase induction, or to regulate a subset of nuclear genes in a more general pathway of communication between mitochondria and the nucleus. The success of the mutagenic screen for regulatory elements is most likely due to the use of the tyrosinase reporter system and the use of the model organism *N. crassa*. Another possible approach would be to screen micro-arrays generated from non-induced and induced cultures to identify differentially expressed transcripts. However, there is no assurance that alternative oxidase regulatory gene products would be differentially expressed – they could instead be differentially localized or activated. Additional future work lies in saturation of our mutagenic screen and in identification of the genes that we have mutated, which should be facilitated by the mapping of the mutations. Once we have identified one or more of the genes that regulated alternative oxidase production, the genes could be analyzed for expression differences in inducing and non-inducing conditions. This would reveal whether or not a microarray approach could be useful in isolating future mutants.

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